









throp and Kunitz (8). The results of eight experiments (1) with serum albumin (crystallized three times) are in complete harmony with the theory of von Smoluchowski and of Henry just published. The ratio  $\frac{U_m}{v_m}$  is very close to  $-1.0$ , as previously reported.

TABLE I

Medium	pH	$v_m$ $\mu/\text{sec.}/\text{volt}/\text{cm.}$	$U_m$	$\frac{U_m}{v_m}$
.....	4.04	1.39	1.52	1.09
.....	3.13	2.26	2.26	1.00
.....	3.76	1.90	2.10	1.11
.....	3.47	2.17	2.26	1.04
ate buffer.....	3.64	1.42	1.22	0.86
ate buffer.....	3.72	1.34	1.66	1.24
ate buffer.....	4.02	1.01	1.02	1.01
ate buffer.....	4.30	0.63	0.61	0.97
can.....				1.04
verage deviation.....				0.08

## SUMMARY

As previously found experimentally for crude protein surfaces, and in harmony with recent theory and experiment, the ratio of the electroosmotic and electrophoretic mobility for surfaces of purified protein is approximately  $-1.00$ .

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# STUDIES IN RESPIROMETRY

## I. A COMBINED GAS BURETTE-INTERFEROMETER RESPIROMETER

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In developing a field of investigation concerned with the influence of radiations on physiological processes<sup>1, 2</sup> we have had as one objective in this laboratory the observation of possible effects of irradiation upon metabolic activity. The study of respiratory rates under various experimental conditions appeared as a likely procedure, but the methods available for such observations seemed unsatisfactory. Accordingly, it was necessary to construct an apparatus for the purpose; and at the suggestion of Dr. Van Name, of the Department of Physical Chemistry, Yale University, the possibility of employing an interferometer for measurement of carbon dioxide changes was investigated.

Considerable information as to the use of an interferometer in gas analysis has been given in two articles<sup>3, 4</sup> by J. D. Edwards, including an excellent method of calibration. It is obvious, however, from a mere perusal of these articles that in the case of gaseous systems in which both oxygen and carbon dioxide concentrations are subject to *independent* change the observation of the resultant change in refractivity does not suffice for the estimation of either of these changes. For the case of fixed pressure ( $p$ ) and temperature ( $T$ ) and a normal mixture of dry CO<sub>2</sub>-free air at the start, however, Edwards<sup>4</sup> has given a method of estimation of these concentration changes ( $\Delta C_{CO_2}$  and  $\Delta C_{O_2}$ ) when in addition to the above we observe the change in refractivity when the carbon dioxide of the system is absorbed finally. The

<sup>1</sup> Hussey, R., Thompson, W. R., and Calhoun, E. T., *Science*, 1927, **66**, 65.

<sup>2</sup> Tennant, R., *Science*, 1931, **73**, 567.

<sup>3</sup> Edwards, J. D., *Bureau Standards Bull.*, 1917, **14**, 473.

<sup>4</sup> Edwards, J. D., *Bureau Standards Technol. Papers*, 1919, **12**, Paper No. 13.

method of calculation given is one of successive approximation; but, as will be seen below, this may be replaced by direct calculation, the desired estimates being expressible as rational algebraic functions of  $p$ ,  $T$ , and the observed changes in refractivity; and, if the actual changes in moles of  $O_2$  and  $CO_2$  are required for a closed system, then the initial volume of the system is the only additional quantity which must be known.

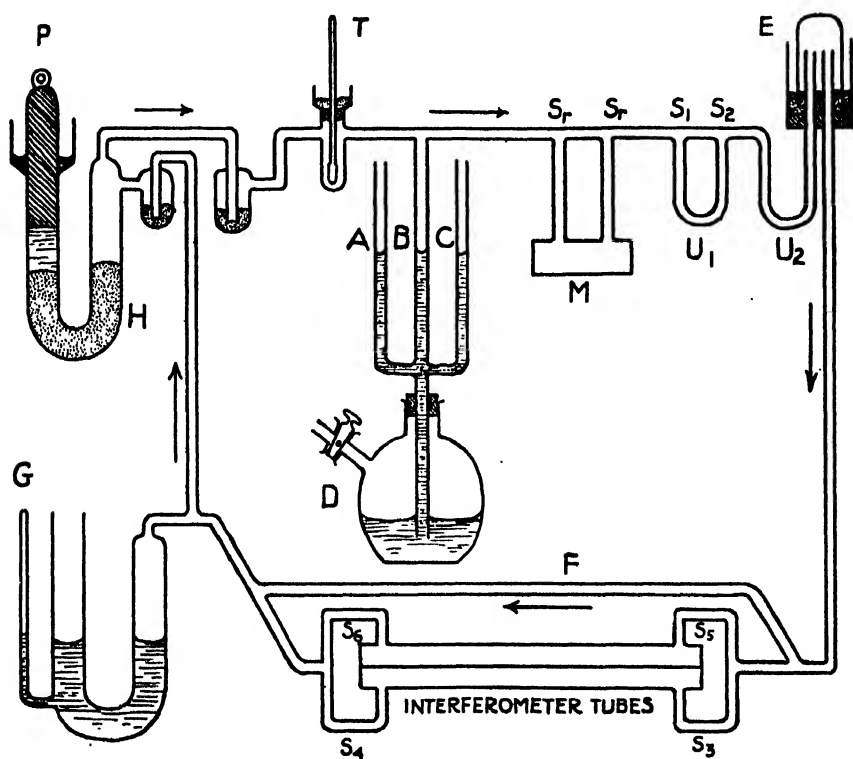
In accord with these principles a machine could be constructed, consisting of a sealed gas chamber (leakage through rubber joints being prevented by some means such as mercury, oil or vaseline seals) including a chamber for the respiring system, a suitable means of circulating the gas,  $CO_2$  and water absorbing tubes introducible at will, and an interferometer of the laboratory type described in the article<sup>4</sup> mentioned above, fixed so that circulation of the gases through either the left, the right or both chambers of the interferometer could be controlled by pairs of stop-cocks (one at each end of each of the tubes) combined with a means of adjusting the pressure of the gas and a means of short circuiting the respiratory chamber proper during absorptions when required, and, of course, some means of introduction of fresh air at will.

Now, as stated above, with such a machine the change of refractivity from the start with dry  $CO_2$ -free air to the end of a given interval ( $t'$ ,  $t''$ ) under conditions of constant temperature and the same pressure at the beginning and end is insufficient for the estimation of either of the corresponding concentration changes or, for a known initial gas volume ( $V'$ ), the change in amount of  $CO_2$  or  $O_2$  (which we shall represent in the following discourse by  $'\Delta''Q_3$  and  $'\Delta''Q_2$ , respectively); but it does give a linear relation between these quantities. Obviously, if another such independent relation could be established, the simultaneous solution of these two linear relations would give the required estimates of  $\Delta Q_3$  and  $\Delta Q_2$  and make our dependence upon data from absorption unnecessary.

The change in volume ( $'\Delta''V$ ) of this system in the interval ( $t'$ ,  $t''$ ), if known, would give just such a relation—if the simple gas laws hold, 
$$' \Delta''V = \frac{22.4 \cdot T}{273 \cdot p} (' \Delta''Q_2 + ' \Delta''Q_3).$$
 Accordingly, we need only a means of precisely estimating this change in volume, for which a gas burette

combined with a delicate indicator of pressure difference between the internal gases and the atmosphere would serve provided that either this atmospheric pressure be sensibly constant or a suitable correction to crude observation be made. Furthermore, the insertion of such a burette into the machine system described above would furnish independent means of checking all of the results.

In Text-fig. 1 is given a schematic diagram of such a machine which has been developed in this laboratory. At *H* there is a pump whose



TEXT-FIG. 1

piston (*P*) runs in paraffin oil above mercury in a U-shaped glass tube, the other side of which is a part of the closed gas system (*S*) of the respiratory machine, and is provided with mercury trap valves at its inlet and outlet tubes, the stroke of the pump and the number of strokes per minute being adjustable.<sup>5</sup> To avoid loss of pumping

<sup>5</sup> The piston of the pump and the piston box were made of gun-metal, the latter being provided with a cup above and attached with rubber tubing to glass tubing

efficiency the valves are sealed to the oil-mercury pump tube in a compact form. It has been found convenient to use Pyrex glass almost throughout the respiratory apparatus except for stop-cock plugs and parts of the interferometer, connections being fused together except for occasional rubber joints sealed with mercury, or, where this is not feasible as in the case of the joints to a metal interferometer tube, rubber tubing completely coated with vaseline has been used. Thus the outlet tube of the pump is joined to a gas burette, *B*, where the gas is held over paraffin oil, the pressure of which may be adjusted delicately to that of the atmosphere by increase or decrease of air pressure in a communicating oil reservoir (the stop-cock *D* and delivery tube being used for this purpose). The two communicating tubes, *A* and *C*, of the same bore and with their axes in the same vertical plane as in the case of the burette, *B*, furnish a means of indicating when the pressure in the closed system, *S*, is the same as the external atmospheric pressure.

Next in the line of flow of the gas is interposed a suitable respiratory chamber (*M*) connected so that by means of a single stop-cock or pair of stop-cocks (*S*<sub>7</sub>) the chamber may be sealed from the system and an alternate *short circuit* automatically made. The inlet and outlet tubes of *M* are joined with short rubber tubing connections, both sealed with a single mercury cup to facilitate connection and disconnection of the chamber. In the same manner the absorption tube, *U*<sub>1</sub>, is connected with stop-cock and short circuit system (*S*<sub>1</sub> and *S*<sub>2</sub>) immediately following *M* in the line of flow and followed by the absorption tube, *U*<sub>2</sub>, through which, however, all gas must pass in circulation. In certain experiments to be described below *U*<sub>1</sub> contained soda-lime and *U*<sub>2</sub> was charged with CaCl2; but, as will be seen later this arrangement may be varied according to whether the living systems are to be kept in dry or moist air and what absorption observations are to be made if any. Next in line we have a vent, *E*, which may be opened or closed merely by removal or replacement of a glass bell from a mercury

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below (at a point such that oil but not mercury could come in contact with metal parts). The author wishes to acknowledge his indebtedness to Mr. Edmund W. Baggott for his suggestions as to the design of the pump, to Dr. Robert Tennant for his criticism and assistance, and to Mr. E. F. Barringham for practical suggestions.

basin in the middle of which stand an inlet and outlet tube to and from the rest of the system, *S*. When fresh air is to be introduced to *S*, a pipe line is connected to the proper one of these tubes so as to draw air directly from the outside of the building into the otherwise closed system, the washed out gases being expelled at the companion tube of the vent. Following the vent system, *E*, leads convey the gases back to the pump; but connected in parallel with this line (*F*) are the interferometer tubes; the right and left sides being fitted, respectively, with the pairs of stop-cocks, (*S*<sub>3</sub> and *S*<sub>4</sub>) and (*S*<sub>5</sub> and *S*<sub>6</sub>), by means of which they may be cut out (either or both) at will.

Inserted between the interferometer tubes and the pump in the line, *F*, is a U-tube (*G*) partly filled with oil which serves to prevent undue pressure variation as one end is in the system, *S*, and the other is exposed to atmospheric pressure with the oil seal between. In the arm of this U-tube remote from *S* is sealed a communicating tube at a point below the oil level at all times which serves as an indicator of mean pressure at this point as the upper end of this tube (safely above the oil level) is provided with only a small vent. This U-oil-tube and the *ABC* burette system act in harmony, one on each side of the pump, to reduce pressure fluctuation in the main part of the gas system. A thermometer, *T*, graduated in tenths of a degree Centigrade, is inserted into the lead from the pump to *B*.

In order that the volume change observations be less influenced by temperature variation, the machine described above is kept in a thermoregulated room. The thermoregulation of a room for such purposes has been regarded fairly generally as a difficult and expensive undertaking; but, for the required purposes, our experience has shown this to be unnecessary. However, the thermoregulation system adopted in the present instance does depend to a slight extent upon manual operation of the air intake vent of the room and upon a supply of cool outside air.

The essential principle employed is that of a main and a differential toluene-mercury thermoregulator operating so that the main thermoregulator throws (*via* a mercury switch) a lamp in parallel with the main heating units, this lamp being immersed in a cylinder filled with oil in which is also one leg of the differential thermoregulator which is in the stream of air from the room and air intake vent passing the inter-



posed main heating units which are thrown on and off by this differential thermoregulator in the usual manner *via* a mercury switch. In addition an auxiliary fan, forcing additional air through the vent into the room when in operation, may be used; current being supplied to it from the same source as used for the main heating units in such manner that when current is passing through these units the speed of the fan is reduced in consequence of the short circuiting. Such an auxiliary fan system has been used with the additional feature that its circuit is completely broken when the main thermoregulator is at a temperature below its trip-point by means of the same relay used for the auxiliary lamp but with a separate mercury break.

Before proceeding further with a description of technical details, it may be expedient to develop the formulae required for the estimations of change in amount and concentration of carbon dioxide and oxygen from the observed data; particularly as these formulae appear remarkably simple in view of the apparent complexity of the system involved. The method of development is to consider first a given ideal situation and later to examine how the developed formulae may be generalized so as to cover other cases.

### *Development of Refractovolumetric Respirometry Formulae*

Consider the case of a gaseous system,  $S$ , consisting of a mixture of nitrogen, oxygen, and carbon dioxide under ideal conditions wherein the simple gas laws ( $p \cdot v_m = \tilde{R} \cdot T$ ) hold, where the total pressure in atmospheres,  $p$ , is constant as is the absolute temperature,  $T$ ;  $\tilde{R}$  being the molecular gas constant, and  $v_m$  the volume of a mole of gas under such conditions (in liters). In general, throughout the present discourse, let the unit of volume be the liter, the unit of pressure be the atmosphere (equivalent to 760 mm. of mercury under the conventional conditions), the unit of temperature be the degree Centigrade, the unit of amount of gas be the mole (of concentration the mole per liter), and the unit of time be the minute; unless otherwise specified. Furthermore, as we are to consider the values of the variables involved at a sequence of instants in time (in chronological order), let us adopt the following convention:

(1) If  $X$  is any one of the variables involved, let  $X^{(k)}$  denote its value at the  $k$ -th instant, and let  ${}^a\Delta^\beta X = X^{(\beta)} - X^{(a)}$ , the increment in  $X$  from the  $\alpha$ -th to the  $\beta$ -th instant.

(2) For the sake of brevity let the prime, double prime, etc., be used to replace these superscripts wherever convenient; thus  $'\Delta''X = X'' - X'$ ,  $'\Delta'''X = X''' - X'$ , and  $''\Delta'''X = X''' - X''$ .

(3) Now, for any variable,  $X_i$ , let

the subscript 1 refer to  $N_2$  gas of the system,  $S$ ,  
 " " 2 " "  $O_2$  " " " " ,  
 " " 3 " "  $CO_2$  " " " " , and  
 " " 0 " " the total gas of the system.

(4) Accordingly, let  $V$  be the volume of the system at a given instant of time,  $t$ ; and  $Q_i$  be the amount of the gas,  $i$ , in the system at that instant, and  $C_i$  be its concentration. Then

$$(5) \quad C_i = \frac{Q_i}{V}, \text{ by definition; and}$$

$$(6) \quad Q_0 = \sum_{i=1}^{i=3} Q_i \quad \text{and} \quad C_0 = \sum_{i=1}^{i=3} C_i.$$

Now, we have assumed  $p$  and  $T$  to be constant, hence

$$(7) \quad C_0 = \frac{1}{v_m} = \frac{p}{R \cdot T} = \frac{273 \cdot p}{22.4 \cdot T} \text{ which is constant;}$$

hence, for any given increment of time, we have

$$(8) \quad 0 = \Delta C_0 = \sum_{i=1}^{i=3} \Delta C_i, \text{ whence } -\Delta C_1 = \Delta C_2 + \Delta C_3.$$

Furthermore, we provide that the amount of nitrogen gas in the system remain constant, that the system initially (*i.e.*, at  $t'$ ) be free from  $CO_2$ , and the ratio of the nitrogen to total gas concentration be 0.7906, the value for so called *normal dry  $CO_2$ -free air*; *i.e.*,

$$(9) \quad \Delta Q_1 = 0 \text{ always; } Q_1' = 0; \text{ and } C_1' = 0.7906 \cdot C_0'.$$

Now, we may let  $C = C_0^{(k)}$ ; and (except for emphasis) we may drop the subzero and the single prime (*i.e.*, let  $X = X_0 = X' = X_0'$  for any variable). Then (6) and (9) give  $C_2' = 0.2094 \cdot C$ ; and

$$(10) \quad \Delta Q = \Delta Q_2 + \Delta Q_3 \text{ always (as } \Delta Q_1 = 0),$$

whence

$$' \Delta'' Q_2 + ' \Delta'' Q_3 = Q'' - Q' = C_0 \cdot ' \Delta'' V,$$

obviously; which relation has been mentioned previously in the introduction.

Now, in accord with the theory of refractivity of gases as outlined by Edwards,<sup>4</sup> we assume that for each of the gases or gaseous mixtures (in fixed proportion) there exists a constant,<sup>6</sup>  $R_i$ , such that  $C_i R_i$  is the refractivity of the gas,  $i$ , at the given concentration; and the refractivity of the mixture at the  $k$ -th instant is  $C_o^{(k)} \cdot R_o^{(k)} = C_o R_o^{(k)}$ ; and

$$(11) \quad C_o R_o^{(k)} = C_1^{(k)} \cdot R_1 + C_2^{(k)} \cdot R_2 + C_3^{(k)} \cdot R_3;$$

where, obviously,  $C_o$ ,  $R_1$ ,  $R_2$ , and  $R_3$  are constants but  $R_o^{(k)}$  depends upon the proportions of the three component gases in the mixture. Then, dropping the subzero, for a given interval

$$(12) \quad C \cdot \Delta R = \sum_{i=1}^{i=3} R_i \cdot \Delta C_i.$$

Now, for convenience, let  $r^{(k)}$  be the refractivity of the gaseous mixture in  $S$  at the  $k$ -th instant, then in (12)  $C \cdot \Delta R = \Delta r$  and by (8) and (12), we have

$$(13) \quad \Delta r = \Delta C_3(R_2 - R_1) + \Delta C_3(R_3 - R_1),$$

where  $\Delta r$  is the observed change in refractivity.

Now, consider the interval ( $t'$ ,  $t''$ ); where  $Q_3' = 0$ ,  $Q_1' = V' \cdot C_1' = 0.7906 \cdot C \cdot V'$ , and  $Q_2' = 0.2094 \cdot C \cdot V'$ . Then

$$(14) \quad \left\{ \begin{array}{l} {}'\Delta''Q_3 = V'' \cdot C_3'' = V'' \cdot {}'\Delta''C_3 \text{ and} \\ {}'\Delta''Q_2 = V''C_2'' - V'C_2' = V'' \cdot {}'\Delta''C_2 + C_2' \cdot {}'\Delta''V \\ \quad = V'' \cdot {}'\Delta''C_2 + 0.2094 \cdot C \cdot {}'\Delta''V, \end{array} \right.$$

whence, (13) gives

$$(15) \quad V'' \cdot {}'\Delta''r = ({}'\Delta''Q_2 - 0.2094 \cdot C \cdot {}'\Delta''V)(R_2 - R_1) + {}'\Delta''Q_3(R_3 - R_1).$$

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<sup>6</sup> The values of  $R_1$  here will be 22.4 times the corresponding values (the *ideal values*, of course) employed by Edwards<sup>3,4</sup>, as we employ the mole per liter as unit of concentration rather than the concentration of a perfect gas at normal conditions of temperature and pressure (760 mm. of mercury at 0°C.).

and by (10)

$$' \Delta'' Q_2 + ' \Delta'' Q_3 = C \cdot ' \Delta'' V.$$

Under (15) we have two linear independent equations (as  $R_2 - R_1$  is negative and  $R_3 - R_1$  positive as will be seen) where all the quantities but  $' \Delta'' Q_2$  and  $' \Delta'' Q_3$  may be evaluated separately. Thus we may solve simultaneously for these two quantities, obtaining the relation

$$(16) \quad ' \Delta'' Q_3 = \frac{V'' \cdot ' \Delta'' r - 0.7906 \cdot C(R_2 - R_1) ' \Delta'' V}{R_3 - R_2},$$

where we have from the data of Edwards<sup>4, 6</sup>

$$\begin{aligned} R_1 &= 22.4(2971) 10^{-7}, \\ R_2 &= 22.4(2704) 10^{-7}, \text{ and} \\ R_3 &= 22.4(4467) 10^{-7}. \end{aligned}$$

The change in amount of oxygen is readily estimated from the calculated value of  $' \Delta'' Q_3$  in (16) by the relation,  $' \Delta'' Q_2 = -' \Delta'' Q_3 + C \cdot ' \Delta'' V$ . Obviously, these estimates are independent of any absorption of  $\text{CO}_2$ , and the formulae may be extended readily to include the case where respiration in successive intervals is to be observed without change to fresh air; and to the case where other gases are present but in fixed concentration. In this last case no difference is needed in the formulae whatever, except that  $p$  is interpreted as the sum of the partial pressures of oxygen, nitrogen, and carbon dioxide, and  $C$  is the sum of the concentrations of these three gases but not of all gases as in the simple case above. The demonstration is obvious in view of the fact that all refractivity difference measurements will be the same as if the additional gases (present in constant concentration) were absent and the total pressure were equal to  $p$ . Furthermore, if we have any other gaseous mixture present in constant amount instead of nitrogen, all that we need do is substitute the corresponding coefficient of refractivity of the mixture for  $R_1$  throughout the formulae and replace 0.7906 by the ratio,  $\phi$ , of the concentration of this gaseous mixture to the sum of this concentration and that of oxygen initially (as in the case of a different initial proportion of nitrogen).

Now, consider the case where all the variables are the same as in

the simple case above at  $t'$  and  $t''$ ; but that the following interval ( $t''$ ,  $t'''$ ) is restricted to a complete absorption of  $\text{CO}_2$  without change in  $Q_1$  and  $Q_2$ . Then

$$(17) \quad Q_1''' = Q_1' = 0, \text{ and } \Delta''Q_1 = Q_1'' = -\Delta'''Q_1 = -\Delta'''Q_2 = -C \cdot \Delta'''V;$$

which with (10) furnishes a means of estimation of change in the amounts of  $\text{O}_2$  and  $\text{CO}_2$  based upon volumetry alone.

We turn, now, to the development of formulae for this purpose based upon interferometry alone; employing the same case as above. Obviously,  $\Delta''Q_2 = \Delta'''Q_2 = C \cdot \Delta'''V$ ; and by (13) and (17) as  $\Delta'''C_2 = 0$ , we have

$$(18) \quad \Delta'''r = \Delta'''C_2(R_2 - R_1)$$

whence

$$\begin{aligned} (19) \quad \Delta''Q_2 &= \Delta'''Q_2 = V''' \cdot \Delta'''C_2 + C_2' \cdot \Delta'''V \\ &= (V' + \Delta'''V) \frac{\Delta'''r}{R_2 - R_1} + 0.2094 \cdot C \cdot \Delta'''V \\ &= \frac{V' \cdot \Delta'''r}{R_2 - R_1} + \Delta''Q_2 \left( 0.2094 + \frac{\Delta'''r}{C(R_2 - R_1)} \right), \end{aligned}$$

whence

$$(20) \quad \Delta''Q_2 = \frac{V' \cdot \Delta'''r}{0.7906(R_2 - R_1) - \frac{\Delta'''r}{C}}$$

whence

$$- \Delta''Q_2 = \frac{V' \cdot \Delta'''r}{0.0004728 + \frac{\Delta'''r}{C}} \text{ (approximately).}$$

Now, by (16), replacing  $C \cdot \Delta'''V$  by its equal in (10) we have, as  $V'' = V' + \Delta'''V$ ,

$$(21) \quad \Delta''Q_2 = \frac{V'' \cdot \Delta'''r - (\Delta''Q_2 + \Delta''Q_2)(0.7906)(R_2 - R_1)}{R_2 - R_1}$$

and by (20) we have

$$\begin{aligned}
 (22) \quad 0.7906 (R_2 - R_1) \cdot {}'\Delta''Q_2 &= V'' \cdot {}'\Delta'''r - {}'\Delta''V \cdot {}'\Delta'''r + {}'\Delta''Q_2 \cdot \frac{{}'\Delta'''r}{C} \\
 &= V'' \cdot {}'\Delta'''r - \frac{{}'\Delta'''r}{C} ({}'\Delta''Q_2 + {}'\Delta''Q_2 - {}'\Delta''Q_2),
 \end{aligned}$$

whence (21) gives

$$(23) \quad {}'\Delta''Q_2 = \frac{-V'' \cdot {}'\Delta'''r + {}'\Delta''Q_2 \left[ \frac{{}'\Delta'''r}{C} - 0.7906 (R_2 - R_1) \right]}{R_2 - R_1},$$

whence

$$\begin{aligned}
 (24) \quad {}'\Delta''Q_2 &= \frac{-{}'\Delta'''r \cdot V''}{R_2 - R_1 + 0.7906 (R_2 - R_1) - \frac{{}'\Delta'''r}{C}} \\
 &= \frac{-{}'\Delta'''r \cdot V''}{0.003476 - \frac{{}'\Delta'''r}{C}} \text{ (approximately).}
 \end{aligned}$$

Now, we may express  ${}'\Delta''Q_2$  by (22) in the form

$$(25) \quad {}'\Delta''Q_2 = \frac{\left( V'' - \frac{{}'\Delta''Q_2}{C} \right) \cdot {}'\Delta'''r}{0.7906 (R_2 - R_1)},$$

$$\text{and} \quad 0.7906 (R_2 - R_1) = -0.0004728$$

$$\text{and} \quad R_2 - R_1 = 0.003949 \text{ (approximately).}$$

The relations (24) and (25) are companions in that they involve  $V''$  as the only volume measurement. In a similar manner we may obtain the companion to (20),

$$(26) \quad {}'\Delta''Q_2 = \frac{-{}'\Delta'''r \left( V' + \frac{{}'\Delta''Q_2}{C} \right)}{R_2 - R_1 + 0.7906 (R_2 - R_1) - \frac{{}'\Delta'''r}{C}},$$

which involves only  $V'$  volumetrically. This possibility has been mentioned in the introduction; the choice between the two pairs of

relations to be used is a matter optional to the observer. This method of estimation (based essentially upon interferometry, and independent of measurement of volume change) will be designated as *the first method*; that based on (16) and (10) (independent of absorption data) will be called *the second method*; and that based upon volumetry alone will be called *the third method*. Obviously, the *first* and *third* methods are independent.

The practical application of the first and third methods involves, however, the taking of a sample of the gas at  $t''$  for the absorption, for which the stop-cock (or stop-cocks) at  $S$ , serve by merely excluding the chamber,  $M$ , and taking the remainder of  $S$  as sample. For the data required in the first method, the volume of this sample ( $v''$ ) is of no significance; but for the third method both  $V''$  and  $v''$  must be estimated as the ideal  $''\Delta'''V$  is calculated from the corresponding change in the volume,  $v$ , of the sample by

$$(27) \quad ''\Delta'''V = ''\Delta'''v \cdot \frac{V''}{v''}.$$

Now, let  $x$  be the reading at a given instant of the gas burette,  $B$ , in liters ( $\frac{dx}{dV} = 1$ ), it being understood that whenever readings are taken the pump piston is to be in a conventionally fixed position (e.g., that of minimum pump gas volume—the lowest point of the piston stroke) and the gas of the inclosed system adjusted to the fixed pressure,  $p$ . Then, obviously, for any given system,  $V - x$  is invariant; and if  $m$  is the volume in liters of the gas space within the chamber,  $M$ , and its connecting tubes to the stop-cock,  $S$ , we may define  $v$  by

$$(28) \quad v = V - m;$$

and, obviously,  $v - x$  is invariant also.

Now, let these two invariants be called  $V_0$  and  $v_0$ , respectively. Then  $V = V_0 + x$  and  $v = v_0 + x$ . Obviously, if  $m$  is known by separate calibration, we need only to know  $v_0$  and the successive values of  $x$  ( $x'$ ,  $x''$ , and  $x'''$ ) in order to calculate all the required volumes and changes in volume. Accordingly, an estimation of  $v_0$  is of prime importance.

### Calibration

In the calibration of  $v_0$ , the vessel ( $M$ ) was replaced by another vessel ( $M'$ ) whose volume plus that of the leads to  $S_r$  was estimated as  $m' = 1.197$  liters (the volume of the leads being estimated as 0.044 liter by calibration, and that of the chamber as 1.153 liters by calibration with water). The whole gas space in  $S$ ,  $U_1$ , and the left side of the interferometer system (the right being already included in  $S$ ) was filled with dry  $\text{CO}_2$ -free air ( $U_1$  and  $U_2$  being charged, respectively, with soda-lime and dry  $\text{CaCl}_2$ ) thoroughly mixed by pumping for 10 minutes. Then the pressure was adjusted (pump in fixed position) as in all readings and  $x'$  read on the burette,  $B$ , and the *zero reading* of the interferometer taken (the mean of at least four interferometer readings always being taken, the scale having been calibrated according to the method of Edwards<sup>3,4</sup>, it being found that over the first ten complete drum revolutions, if  $y$  is the reading in *small scale divisions*, then  $2.00 (10^{-8}) \Delta y$  approximates  $\Delta r$  within tolerable limits). Then the chamber ( $M'$ ) was short circuited at  $S_r$ , as was  $U_1$  at  $S_1$  and  $S_2$ , and  $S_5$  and  $S_6$  closed (isolating the gas in the left interferometer tube), and the gas in the remainder of the system replaced by dry gas containing enough  $\text{CO}_2$  to differ in refractivity enough to be measurable with satisfactory precision; mixed with pumping (about the restricted system), and  $x''$  the burette volume and  $\Delta y$  observed by reading the interferometer as before but merely turning the stop-cock,  $S_6$ , through a half revolution (closed-open-closed) in the stagnant system just before each interferometer reading, as difference in pressure between the two sides would cause considerable error and the slight mixing in this way is unimportant. Thirdly, the gas in  $M'$  is mixed as initially with this gas of the right side ( $U_1$  always shut off) and  $x$  and  $y$  observed again. Call the three successive readings of each  $x'$ ,  $x''$ ,  $x'''$  and  $y'$ ,  $y''$ ,  $y'''$  respectively. Then in estimating  $v_0$ , obviously,  $x'$  has no significance except that the pressure is adjusted in the reading and  $x''$  and  $x'''$  will be the same, practically, if temperature and pressure are stable; and  $v_0$  is estimated by

$$(29) \quad v_0 = \frac{m'(y''' - y')}{y'' - y'''} - x''.$$



Thus the values, 0.573, 0.575, 0.566, 0.584, and 0.568 were obtained of which the mean,  $m_{(v_0)} = 0.5732$  and A.D. = 0.0022 which is approximately 0.4 per cent of the mean. The precision was sufficient for immediate purposes; and the approximation,  $v_0 = 0.573$ , has been used ( $V_0 = v_0 + m$ ).

#### EXPERIMENTAL

In order to illustrate the use of the respirometer described above, an experiment upon the respiration of *Drosophila* imagos in dry air at constant temperature, in which all three methods were used and contrasted, will be outlined.

TABLE I

Comparison of three methods of simultaneous estimation of carbon dioxide and oxygen respiration of *Drosophila* imagos in dry air.

Respiration in moles $10^{-6}$ in 1 hr. of approximately 4.54 ( $10^3$ ) flies						
Method .....	Carbon dioxide			Oxygen		
	I	II	III	I	II	III
Exp.						
1	488	478	496	673	749	767
2	464	461	459	636	666	664
3	489	484	481	651	685	682
4	494	494	482	695	686	674
5	485	479	482	641	687	690
Mean .....	484	479	480	659	695	695
Mean* .....	483	480	476	655	681	678

\* Experiment 1 excluded.

Approximately 4.54 ( $10^3$ ) flies were confined in a respiratory vessel,  $M$ , of 0.255 liter capacity; thus, the fly volume being neglected,  $m$  was taken as 0.255 + 0.044 = 0.299 liter, whence  $V_0 = 0.872$  liter. The vessel was covered with black paper throughout five successive sets of observations as follows:

$U_1$  was charged with soda-lime and  $U_2$  with  $\text{CaCl}_2$  (dry). In each set of observations fresh air was introduced by pumping<sup>7</sup> for 5 minutes with vent open and intake connected to a funnel at the window (plugged with absorbent cotton), the circulation being through all parts of the machine except  $U_1$ . Then the air was similarly circulated<sup>7</sup> for 10 minutes with the vent closed and through  $U_1$  which was sufficient to remove sensibly all  $\text{CO}_2$  and moisture (the position of the

<sup>7</sup> The pumping rate was approximately 0.72 liter per minute.

gas in the burette,  $B$ , being adjusted at the moment when the vent was closed so that there was always plenty of room for anticipated expansion or contraction). The end of this interval was taken as  $t'$ , the volume and refractivity readings made,  $S_1$  and  $S_2$  turned so as to short circuit  $U_1$ , and the left interferometer tube isolated by closing  $S_5$  and  $S_6$  (stop-cocks never being turned unless the gas is not circulating and adjusted at  $B$  to atmospheric pressure). Then circulation was recommenced and continued to a time,  $t''$ , such that  $t'' - t' = 60$  minutes. Observations were made then as before and the chamber,  $M$ , isolated at  $S_7$ ,  $U_1$  reconnected in circulation (by  $S_1$  and  $S_2$ ), and pumping continued for 10 minutes at which time,  $t'''$ , another observation of  $x$  and  $y$  was made. Thus we obtained from  $x'$ ,  $x''$ ,  $x'''$  and  $y'$ ,  $y''$ ,  $y'''$  the required data (as  $v = v_0 + x$ , and  $V = V_0 + x$ ) for estimation of  $'\Delta''Q_3$  and  $'\Delta''Q_2$  by each of the three methods. The barometric pressure was estimated (to 0.05 mm. Hg) and the internal temperature (to 0.01°C.) in each instance and suitable correction introduced. The calculated results are given in Table I.

#### DISCUSSION

The corrections for temperature and barometric pressure variation which are usually needed in computing the ideal volume changes can be applied with high precision when the machine is kept in a thermo-regulated room (as indicated above) in which is kept a precision-barometer, read frequently enough to make possible the estimation of  $p$  at any instant during the experience in question to about one part in 10,000; and the need of these corrections serves a useful purpose in making the influence of personal bias upon the volume change observations practically nil. To a high degree of approximation in the usual experience the total correction in a given interval required for  $\Delta V$  may be taken as  $V\left(\frac{\Delta p}{p} - \frac{\Delta T}{T}\right)$ , the exact correction term for the ideal  $'\Delta''V$  at  $p''$  and  $T''$  being  $V' \cdot f_{(1, 2)}$  where

$$(30) \quad f_{(\alpha, \beta)} = \frac{C_o^{(\beta)} - C_o^{(\alpha)}}{C_o^{(\beta)}} = \frac{\alpha_{\Delta}^{\beta} p}{p^{(\beta)}} - \frac{\alpha_{\Delta}^{\beta} T}{T^{(\alpha)}} \cdot \frac{p^{(\alpha)}}{p^{(\beta)}}.$$

In interferometer measurements where the left chamber contains a gas having the same refractivity coefficient as the gas initially in the right chamber and where the temperature and pressure of these two chambers are always equal during reading intervals, the observed increment in refractivity difference ( $\Delta\rho$ ) between the directly compared gases is exactly equal to that ( $\Delta r$ ) which would be found be-

tween the initial and the final gas mixture of the right side were they compared at the final temperature and pressure. Returning to the notation for variable total gas concentration,  $C_o^{(k)}$  introduced before (11); then the observed refractivity difference ( $'\Delta^k\rho$ ) mentioned above is merely  $C_o^{(k)} \cdot '\Delta^k R$ , and  $'\Delta^k R = \frac{'\Delta^k \rho}{C_o^{(k)}}$ , which is independent of variation in  $T$  and  $p$  even when we generalize the meaning of  $\Delta r$  to include actual measured refractivity differences in the above manner. Furthermore, we have the obvious identity,

$$(31) \quad {}^\alpha \Delta^\beta R \equiv '\Delta^\beta R - '\Delta^\alpha R;$$

independent of temperature or pressure variation; and, if  $C$  had been constant throughout the experience and equal to  $C_o^{(\beta)}$ , then we should have observed an ideal refractivity difference,

$$\begin{aligned} (32) \quad ({}^\alpha \Delta^\beta r)_\beta &= C_o^{(\beta)} {}^\alpha \Delta^\beta R = C_o^{(\beta)} \left( \frac{'\Delta^\beta \rho}{C_o^{(\beta)}} - \frac{'\Delta^\alpha \rho}{C_o^{(\alpha)}} \right) \\ &= '\Delta^\beta \rho - '\Delta^\alpha \rho \cdot \frac{C_o^{(\beta)}}{C_o^{(\alpha)}} \\ &= {}^\alpha \Delta^\beta \rho + '\Delta^\alpha \rho \cdot \frac{C_o^{(\alpha)} - C_o^{(\beta)}}{C_o^{(\alpha)}} = {}^\alpha \Delta^\beta \rho + '\Delta^\alpha \rho \cdot f_{(\beta, \alpha)} \end{aligned}$$

which formulae hold regardless of order of observation ( $\alpha \leq \beta$ ). This last term in the last expression, obviously, is the required correction term to observed refractivity differences before insertion in all preceding formulae where  $C_o$  was assumed constant. Obviously, as has been stated, there is no correction required unless  $\alpha \neq 1$ ; and then the limits of tolerance of neglect of the correction are obvious in (32).

Correction for failure initially to obtain air of the required composition in the ideal case may be made in an obvious manner by estimation of probable deviation in refractivity; if this is not negligible. However, it should be noted that the third method is independent of such variation, and for constant (or negligibly variant) temperature and pressure  $\Delta Q_2 + \Delta Q_3$  is estimated in the second method as well as the third independently of the volume of the system. This quan-

tity,  $\Delta Q_o$  or  $\Delta Q = \Delta Q_2 + \Delta Q_3 = \Delta(CV)$  as  $Q = CV$  and  $Q_1$  is constant, may be used to express the fundamental relations of all three methods in general form (where  $p$  and  $T$  may vary) in conjunction with the values of  $\Delta R$  and  $\phi = \frac{Q'_1}{Q}$  (for which the value, 0.7906 has been assumed in the preceding development) as follows:

$$\begin{aligned}
 (33) \quad {}^{\prime}\Delta''Q_3 &= \frac{- {}^{\prime}\Delta'''R \cdot V''C_o''}{R_3 - R_2 + \phi(R_2 - R_1) - {}^{\prime}\Delta'''R} & \text{I} \\
 &= \frac{{}^{\prime}\Delta''R V''C_o'' - \phi(R_2 - R_1) {}^{\prime}\Delta''Q_o}{R_3 - R_2} & \text{II} \\
 &= \frac{- C_o''V_o''({}^{\prime}\Delta'''v + v''' \cdot f_{(3,2)})}{v''} & \text{III}
 \end{aligned}$$

where  $f_{(\alpha,\beta)} = \frac{C_o^{(\beta)} - C_o^{(\alpha)}}{C_o^{(\beta)}}$ , and  ${}^{\alpha}\Delta^{\beta}Q_o = C_o^{(\beta)} ({}^{\alpha}\Delta^{\beta}V + V^{(\alpha)} \cdot f_{(\alpha,\beta)})$ ; and

$$\begin{aligned}
 (34) \quad {}^{\prime}\Delta''Q_2 &= \frac{{}^{\prime}\Delta'''R(C_o'V'' - {}^{\prime}\Delta''Q_3)}{\phi(R_2 - R_1)} & \text{I} \\
 &= {}^{\prime}\Delta''Q_o - {}^{\prime}\Delta''Q_3 & \text{II and III.}
 \end{aligned}$$

It is obvious that no assumption of constant temperature or pressure or constant total concentration,  $C_o$ , need be made in application of these general formulae which hold in the first and second cases (I and II) independently of the holding of the simple gas laws; which, however, are used as a means of estimating  $C_o$  and  ${}^{\prime}\Delta''Q_o$ . The complexity of the forms involving  $f_{(\alpha,\beta)}$  is introduced to facilitate calculation by approximation methods, *e.g.*, by slide rule; and for any given interval  $f$  may be approximated by  $\frac{\Delta p}{p} - \frac{\Delta T}{T}$ , usually with negligible error when either the temperature or the pressure variation is slight. In practice, however, the forms given earlier in the text have been employed with  $\Delta r$  replaced by its equivalent in terms of the directly observed values of  $\Delta y$ .

In regard to leakage it may be stated that there were no detectable leaks anywhere in the machine excepting the interferometer tubes

themselves. The first tube employed in preliminary observations leaked so badly that it could not be used for further work without repair. The second tube was much better and by adjustment of pressure at *D* and observation of mean pressure variation at *G* it was found that leakage could be controlled at will by such adjustment (made either positive or negative). Accordingly, in the experiments reported adjustment was made to approximate a zero resultant leakage as nearly as practicable. The actual leakage rate under such conditions was subsequently estimated as about  $-0.000,01$  liter per minute (A.D. =  $0.000,005$  liter per minute). No correction for this leakage has been applied, however, although its application would have brought the results into closer agreement.

#### SUMMARY

A machine has been described which combines volumetric and refractometric means of observing changes in carbon dioxide and oxygen concentrations and amounts in a closed system suitable for containing a respiring system. A means of thermoregulation has been described briefly. The theory upon which estimation is based has been outlined in considerable detail and generality; indicating how dependence upon absorption of gases by reagents can be eliminated completely, although they may be used in certain instances as a means of independent check. An experiment of this nature has been reported as an illustration of one use of the machine. Data of other experiments in which a moist environment was employed for the respiring system will be given in another communication.

## STUDIES IN RESPIROMETRY

### II. INFLUENCE OF INFRA-RED RADIATION UPON CARBON DIOXIDE RESPIRATION OF DROSOPHILA IMAGOS IN DRY AIR

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In another communication<sup>1</sup> a respirometer has been described which makes possible the simultaneous estimation of carbon dioxide and oxygen change in a closed system, not necessarily dry. However, if the gases are kept dry, there are available independent means of estimating these changes provided that the initial air approximates the composition of so called *normal dry CO<sub>2</sub>-free air*. As it was planned to use *Drosophila* imagos in further work,<sup>2</sup> it seemed of value to estimate the influence of visible and infra-red radiation upon their respiratory rates in order to see what precautions should be taken to prevent confusion of such possible effects with others under investigation as well as to furnish an example of the use of the respirometer in differential measurements.

The arrangement of material in respiration observations was the same as in the experiments described in the report<sup>1</sup> mentioned above excepting the presence of a source of radiation and a means of its direction, filtration, or screening as stated below. Under such conditions it might be expected that volumetric measurements be somewhat less reliable than in the simpler case first examined, but the influence of such errors upon the estimation of CO<sub>2</sub> changes is very slight as will be seen in the following results where irradiation consisted of merely turning on an adjacent reading lamp as well as in the first experiment of the previous report.<sup>1</sup> The results by what has been designated there as *the first method* should not be affected greatly by this disturbance, however, hence the decision to work with dry air at

<sup>1</sup> Thompson, W. R., *J. Gen. Physiol.*, 1932-33, 16, 5.

least in preliminary investigation—a comparison in moist air to be made later.<sup>2</sup>

In order to ascertain whether effects of irradiation were of a temporary nature or not, a procedure of alternation of observations of respiration (over intervals of an hour) with and without irradiation

TABLE I

Influence of irradiation with visible and infra-red radiation from a reading lamp (60 watt) at approximately 24 cm. upon respiration of *Drosophila* imagos.

Respiration in millimoles per hr.							Respiratory quotient		
Carbon dioxide				Oxygen					
Method.. . . . .	I	II	III	I	II	III	I	II	III
<i>D</i>	0.50	0.50	0.42	0.75	0.74	0.66	0.67	0.68	0.64
<i>L</i>	0.69	0.70	0.66	0.95	0.85	0.82	0.73	0.82	0.81
<i>D</i>	0.52	0.51	0.50	0.71	0.73	0.72	0.73	0.70	0.69
<i>L</i>	0.68	0.69	0.68	0.95	0.84	0.83	0.72	0.82	0.82
<i>D</i>	0.50	0.50	0.51	0.75	0.73	0.74	0.67	0.69	0.69
<i>D</i>	0.53	0.55	0.53	0.88	0.77	0.75	0.60	0.71	0.71
<i>L</i>	0.85	0.87	0.88	1.15	0.99	1.00	0.74	0.88	0.88
<i>D</i>	0.56	0.57	0.58	0.83	0.77	0.78	0.67	0.74	0.74
<i>L</i>	0.81	0.83	0.83	1.07	0.91	0.91	0.76	0.91	0.91
<i>D</i>	0.54	0.54	0.57	0.76	0.76	0.79	0.71	0.71	0.72
<i>D</i>	0.55	0.55	0.55	0.78	0.73	0.73	0.71	0.75	0.75
<i>L</i>	0.83	0.85	0.83	1.09	0.97	0.96	0.76	0.88	0.86
<i>D</i>	0.57	0.57	0.58	0.76	0.77	0.79	0.75	0.74	0.73
<i>L</i>	0.72	0.74	0.73	0.98	0.86	0.86	0.74	0.86	0.85
<i>D</i>	0.46	0.46	0.46	0.67	0.69	0.69	0.69	0.67	0.67
Mean ( <i>D</i> *). . . .	0.529	0.531	0.535	0.768	0.744	0.749	0.689	0.714	0.714
Mean ( <i>L</i> ). . . . .	0.763	0.780	0.768	1.032	0.903	0.897	0.739	0.864	0.856

\* First *D* observation excluded (*D* unirradiated, *L* irradiated).

was adopted. In Table I are given the results obtained (employing the *three methods* of the previous work<sup>1</sup> simultaneously) in five respiratory observations on each of three lots of imagos (approximately 5.40, 5.07, and 6.08 thousand in aggregate number, respectively—the

<sup>2</sup> Thompson, W. R., and Tennant, R., *J. Gen. Physiol.*, 1932-33, 16, 27.

age since emergence from the pupa case being less than 3.1 days at the beginning of the respiratory observations). Alternately, in each instance, respiration was observed in the dark (*D*), using a black paper cover, and in the light and infra-red radiation (*L*) a 60 watt daylight reading lamp with reflector at a distance of approximately 24 cm. from the objectives being turned on during the interval

TABLE II

Comparative influence upon respiration of *Drosophila* imagos of the visible radiation alone and combined with the infra-red radiation from a 300 watt lamp under given conditions.

Method....	Respiration in millimoles per hr.						Respiratory quotient		
	Carbon dioxide			Oxygen					
	I	II	III	I	II	III	I	II	III
<i>D</i>	0.73	0.72	0.76	0.94	1.00	1.04	0.78	0.72	0.73
<i>L</i> <sub>0</sub>	0.74	0.73	0.76	0.97	1.00	1.02	0.76	0.73	0.74
<i>L'</i>	0.81	0.80	0.86	1.01	1.06	1.12	0.80	0.76	0.77
<i>D</i>	0.81	0.80	0.85	1.01	1.07	1.11	0.80	0.75	0.77
<i>L'</i>	0.75	0.75	0.81	1.00	0.97	1.03	0.75	0.77	0.79
<i>D</i>	0.51	0.50	0.47	0.73	0.81*	0.79*	0.70	0.62*	0.60*
<i>L'</i>	0.49	0.49	0.47	0.68	0.72	0.70	0.72	0.68	0.67
<i>L''</i>	0.55	0.56	0.55	0.80	0.77	0.76	0.69	0.73	0.72
<i>D</i>	0.47	0.48	0.50	0.69	0.66	0.68	0.68	0.73	0.74
<i>L''</i>	0.54	0.54	0.57	0.69	0.69	0.73	0.78	0.78	0.78
<i>D</i>	0.45	0.43	0.46	0.50*	0.61	0.64	0.90*	0.70	0.72

*D* in darkened room; *L*<sub>0</sub> in direct visible radiation; *L'* in direct and reflected visible radiation (three mirrors); *L''* the same as *L'* plus infra-red radiation (water filter removed).

\* Comparison of respiratory quotients indicates unreliability of oxygen values in these instances regardless of correlation between II and III. All observations are included in the table.

(1.7, 55.0) of the total respiratory interval, (0, 60) minutes. This restriction was made in order that the disturbance of temperature control might not be so great as to preclude the desired observations. The increase in respiratory rates with such irradiation (about 2/5) is striking as is the subsequent return to the previous level.

In order to differentiate between effects of visible and infra-red



components of such radiations the following experiments were made, using two lots of imagos (approximately 5.88 and 5.80 thousand in number and of imago age not exceeding 4.1 days). The results of irradiation with and without the infra-red component ( $L''$  and  $L'$ , respectively) throughout the whole of the 60 minute intervals of collection employing a 300 watt lamp with parabolic reflector at a distance of about 1.3 meters from the objectives with triple addition to the direct radiation by the use of three mirrors may be compared with respiration without irradiation ( $D$ ), the room being shielded from all but a small amount of light. These results are given in Table II together with one instance wherein the reflecting mirrors were not used ( $L_0$ ); and in addition are appended in each instance (as in Table I) the so called *respiratory quotients* as calculated according to the data of the three methods employed, the ratio of carbon dioxide increment to minus that of oxygen in the interval, according to convention.

These quotients are interesting in general on account of an apparently great stability under many conditions and variation with diet. Here, they may be used as a basis of choice between conflicting estimates of the same oxygen increments (the  $\text{CO}_2$  estimates usually being in close approximation even under the most trying conditions, particularly in the first and second methods), and may indicate the preference of one value despite a close approximation of the two rival values.

## STUDIES IN RESPIROMETRY

### III. AN APPLICATION OF REFRACTOVOLUMETRIC RESPIROMETRY TO THE OBSERVATION OF CONTINUOUS RESPIRATORY CHANGES IN WET OR DRY SYSTEMS

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(Accepted for publication, May 12, 1932)

The principles of refractovolumetric respirometry have been treated in considerable detail in a previous report,<sup>1</sup> in which was described a machine admitting of use in measuring carbon dioxide and oxygen respiration in either wet or dry systems. In another communication<sup>2</sup> an application has been presented, restricted however to a dry atmosphere. It is the purpose of the present report to demonstrate the facility with which continuous respiratory changes may be observed in either dry or water-saturated environments; using what has been designated *the second method* in the previous work,<sup>1</sup> where the general theoretical considerations are presented.

As a preliminary experiment the respiration of a lot of *Drosophila* imagos was observed in this manner, first allowing a 40 minute interval to elapse during which observations were ignored (as in the case of the wet environment such interval was necessary to insure saturation of the gaseous system with water), and then observing respiration in three successive 20 minute intervals; renewing the air and repeating the process alternately with dry and wet environment. The results of this experiment are given in Table I.

In later work the modification of bubbling the fresh air through water before admission to the system was introduced in experiments requiring a water-saturated system in order to avoid most of the above mentioned delay. Some preliminary equilibration period is necessary

<sup>1</sup> Thompson, W. R., *J. Gen. Physiol.*, 1932-33, 16, 5.

<sup>2</sup> Thompson, W. R., and Tennant, R., *J. Gen. Physiol.*, 1932-33, 16, 23.

however, and correction for respiration during this interval should be introduced. In actual application of the formulae given previously<sup>1</sup> it is obvious that this may be done with tolerable error by a simple extrapolation. The formulae for  ${}^{\alpha}\Delta^{\beta}Q_3$ , the  $\text{CO}_2$  increment, is obtained (in the form invariant with temperature and pressure) from II under Equation (33) of the above mentioned paper<sup>1</sup> by taking the

TABLE I

Respiration of *Drosophila* imagos in wet and dry atmosphere, after 40 minutes, in 20 minute intervals.

	Respiration (in millimoles)*		Respiratory quotient
	Carbon dioxide	Oxygen	
Dry	0.149	0.190	0.78
	0.146	0.212	0.69
	0.138	0.200	0.69
Wet	0.180	0.196	0.92
	0.185	0.249	0.74
	0.190	0.242	0.79
Dry	0.175	0.229	0.76
	0.161	0.239	0.67
	0.172	0.215	0.80
Wet	0.182	0.226	0.81
	0.185	0.229	0.81
	0.184	0.216	0.85

\* Aggregate number of imagos =  $4.67(10)^3$ , approximately.

difference between  $'\Delta^{\beta}Q_3$  and  $'\Delta^{\alpha}Q_3$ , whence (employing the same notation) we have

$$(1) \quad {}^{\alpha}\Delta^{\beta}Q_3 = \frac{{}'\Delta^{\beta}R \cdot V^{(\beta)}C_o^{(\beta)} - {}'\Delta^{\alpha}R \cdot V^{(\alpha)}C_o^{(\alpha)} - \phi(R_2 - R_1) \cdot {}^{\alpha}\Delta^{\beta}Q_o}{R_3 - R_2},$$

whence we have, as  ${}^{\alpha}\Delta^{\beta}Q_o = V^{(\beta)}C_o^{(\beta)} - V^{(\alpha)}C_o^{(\alpha)}$  by definition,

$$(2) \quad {}^{\alpha}\Delta^{\beta}Q_3 = \frac{{}'\Delta^{\beta}R \cdot V^{(\beta)}C_o^{(\beta)} + [{}'\Delta^{\alpha}R - \phi(R_2 - R_1)] {}^{\alpha}\Delta^{\beta}Q_o}{R_3 - R_2}.$$

Similarly, but with less difficulty, we have (from (34) of the first paper<sup>1</sup>) for the oxygen increment

$$(3) \quad {}^{\alpha}\Delta^{\beta}Q_2 = {}^{\alpha}\Delta^{\beta}Q_o - {}^{\alpha}\Delta^{\beta}Q_1.$$

In the case of either wet or dry systems these same formulae apply;  $C_o^{(k)}$  being the sum of the concentrations of  $\text{CO}_2$ ,  $\text{O}_2$ , and the inert gas present in *constant amount*; (e.g., nitrogen), but exclusive of gases present in constant concentration such as  $\text{H}_2\text{O}$  in the case of water-saturated environments;  $R_1$  being the refractivity coefficient of this inert gas, and  $R_2$  and  $R_3$  being that of oxygen and carbon dioxide, respectively (as previously<sup>1</sup> explained); and  $\phi$  the fraction represented by the inert gas (e.g.,  $\text{N}_2$ ) in moles of the original mixture. As explained before<sup>1</sup> superscripts refer to instants in time, and the subscripts refer

1 to the inert gas (present in constant amount)

2 to oxygen

3 to carbon dioxide, and

0 or no subscript to their sum;

and  ${}^{\alpha}\Delta^{\beta}X_i = X_i^{(\beta)} - X_i^{(\alpha)}$ . Thus the variables used above may be defined by the statement in addition to this that  $Q$  is amount of gas in moles,  $C$  concentration in moles per liter,  $R$  the refractivity coefficient of the gas (i.e.,  $R \cdot C$  is the refractivity of the gas) and  $V$  is the volume of the system and  $\phi = \frac{Q_1'}{Q_o'}$  by definition.

Thus two experiments were performed, observing in the first instance continuous respiration of approximately 4.97 thousand imagoes in a dry system in successive intervals of 20 minutes (up to 500 minutes when the amount of accumulated  $\text{CO}_2$  was over one-tenth of all gas present); and in the second observing respiration of approximately 6.88 thousand imagoes in a water-saturated environment in a similar manner (intervals of 10 minutes being used, however). The temperature was  $24.2^\circ\text{C}$ ., approximately (in actual experience in the two experiments temperature observations made at the beginning and end of each interval lay between  $24.16$  and  $24.30^\circ\text{C}$ . in every instance).

The results of the two sets of observations are given in Tables II and III and presented graphically in Text-fig. 1. It is of interest to note the approximation of the respiratory quotients for the whole period of

TABLE II

Respiration of *Drosophila* imagos\* in dry air, initially CO<sub>2</sub>-free, in millimoles in 100 minute intervals.

Time	Carbon dioxide (approx.) total	Oxygen (approx.) total	All gases (approx.) total
<i>min.</i>			
0	0	7.8	37.8
100	0.9	6.5	37.4
200	1.8	5.3	37.1
300	2.6	4.2	36.8
400	3.3	3.3	36.6
500	3.9	2.5	36.4

\* Aggregate number of imagos =  $4.97 (10)^3$ , approximately.

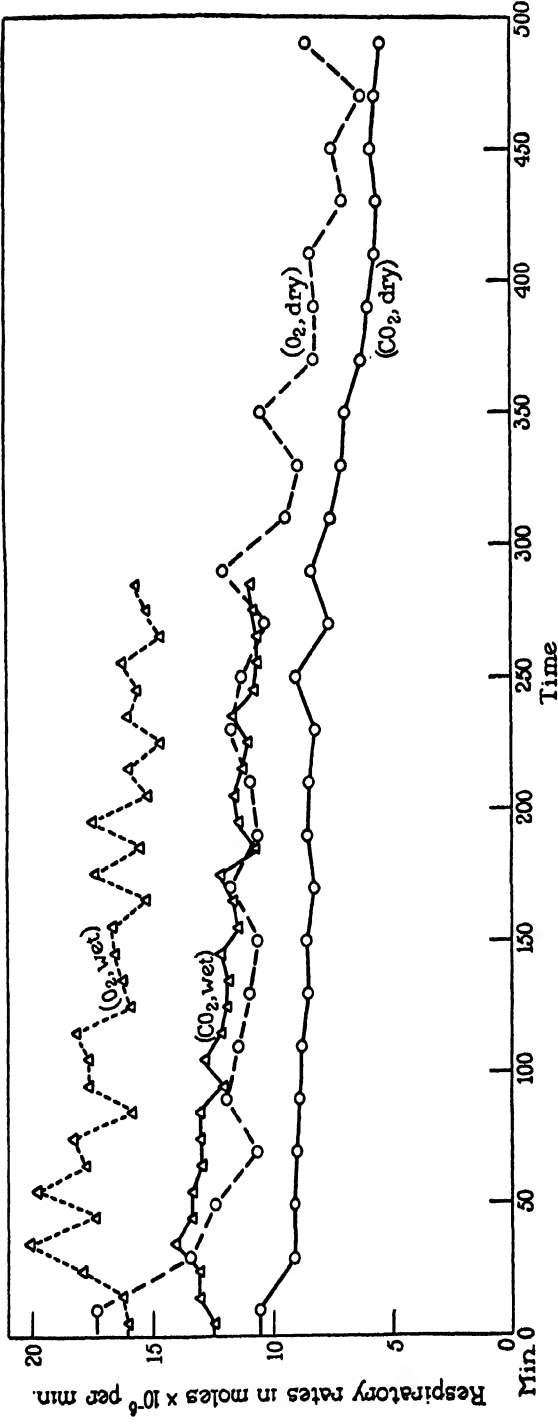
TABLE III

Respiration of *Drosophila* imagos\* in water-saturated air, in millimoles in 100 minute intervals.

Time	Carbon dioxide (approx.) total	Oxygen (approx.) total	All gases except H <sub>2</sub> O (approx.) total
<i>min.</i>			
0	0.1	7.3	35.5
100	1.4	5.5	35.0
200	2.6	3.8	34.6
290	3.6	2.4	34.2

\* Aggregate number of imagos =  $6.88 (10)^3$ , approximately.

observation in the dry and the water-saturated experiences, being 0.741 and 0.722, respectively. In each instance about two-thirds of the original amount of oxygen was consumed.



TEXT-FIG 1.



# CRYSTALLINE PEPSIN

## IV. HYDROLYSIS AND INACTIVATION BY ACID

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Experiments with crystalline pepsin already described (1-3) show that the crystalline protein has a constant and characteristic proteolytic activity. The proteolytic activity of various preparations is the same and cannot be changed by repeated recrystallization, partial solution in various salt concentrations, or by fractional diffusion. The loss of activity when the protein is denatured by alkali corresponds to the amount of denatured protein formed and when this denatured protein is changed back to native protein the original activity is regained. These results render it very improbable that the activity is due to the presence of any non-protein molecule adsorbed on the protein molecule since adsorption complexes in general do not have constant properties. They indicate that the proteolytic activity is a property of this molecular species of protein, just as the peculiar properties of hemoglobin are attributes of the molecule of the protein, hemoglobin. In both cases the characteristic property is lost when the protein is denatured and regained when the protein is again brought into the native form (2, 4). In the case of hemoglobin it is known that the characteristic properties are due to the presence of a special prosthetic group in the molecule and it is probable that the pepsin protein also contains some characteristic group to which its activity is due. There is at present no evidence as to the nature or even the existence of such a characteristic group and, owing to the difficulty of determining the structure in the case of proteins, it is difficult to obtain such evidence by the ordinary methods of structural chemistry. The problem would be greatly simplified if a part of the protein molecule possessing even very slight activity could be split



off. The attempt was therefore made to hydrolyze pepsin under conditions which would not completely destroy the activity and to determine whether a solution could be obtained which was more active than would be expected from its content of protein. If any of the split products of the protein were appreciably active compared to the original protein molecule the total activity of the solution would be higher than that calculated from its protein content.

Unfortunately the methods of hydrolyzing the protein are limited practically to dilute acid. Several attempts were made to hydrolyze pepsin with trypsin at pH 6 which is as far on the alkaline side as it is possible to work without causing alkali inactivation. The trypsin is, however, very rapidly destroyed by the pepsin and no hydrolysis of the pepsin could be detected so that there was no change in either the protein concentration or the activity when trypsin was added to pepsin solutions. Attempts to hydrolyze the protein with papain were also unsuccessful. No hydrolysis of the pepsin protein or decrease in peptic activity could be detected. Most of the protein present in the papain preparation used was very rapidly digested and there was a slight loss in papain activity so that the results indicate that papain is digested by pepsin although very much more slowly than is trypsin.

#### *Hydrolysis in Dilute Acid*

It is known that pepsin solutions lose their activity slowly when allowed to stand in dilute acid and it was shown in the first paper (1) that this loss in activity was exactly equivalent to the amount of native protein destroyed, since if a saturated solution of the protein crystals is partially inactivated and then saturated again with the crystals, the activity of the solution returns exactly to its original value while the total nitrogen content increases to just the extent calculated from this increase in activity. This is a very sensitive method since the solubility is probably the most specific property of a protein. It cannot be used, however, to follow the hydrolysis very far since the products formed by the hydrolysis, when present in considerable quantities, begin to affect the solubility of the crystals. In the present experiments, therefore, the concentration of protein nitrogen in the solution was determined directly by precipitating the protein with 2.5 per cent trichloroacetic acid and also by determining

the quantity of protein which becomes denatured and insoluble when the solution is made alkaline. This effect of alkali is a characteristic property of the pepsin protein and it seemed possible that the figure obtained in this way might differ from that obtained with 2.5 per cent trichloroacetic acid since the latter reagent might precipitate other substances than the unchanged protein itself. However, the results showed that exactly the same amount of protein nitrogen was determined by both methods. In addition, the amount of nitrogen precipitated by phosphotungstic acid was also determined. The loss in activity was determined by several methods.

TABLE I

*Decrease in Activity and Protein Nitrogen in Pepsin Solutions at Different pH*

5 gm. three times crystallized pepsin stirred with 50 ml. H<sub>2</sub>O and dissolved by addition of M/5 NaOH. 5 ml. of this solution added to 20 ml. various concentrations HCl; solution kept at 35°C. and analyzed for protein nitrogen (2.5 per cent CCl<sub>3</sub>COOH) and activity.

Concentration HCl mols/l..	0.01			0.05			0.20			1.0			2.0		
Approximate pH.....	4.0			1.5			0.7			0			+0.3		
Hrs. at 35°C.	P.N./ml. mg.		[P.U.] ml.	P.N./ml. mg.		[P.U.] ml.	P.N./ml. mg.		[P.U.] ml.	P.N./ml. mg.		[P.U.] ml.	P.N./ml. mg.		[P.U.] ml.
	Gel. V <sup>-</sup>	Ed. V <sup>-</sup>		Gel. V <sup>-</sup>	Ed. V <sup>-</sup>		Gel. V <sup>-</sup>	Ed. V <sup>-</sup>		Gel. V <sup>-</sup>	Ed. V <sup>-</sup>		Gel. V <sup>-</sup>	Ed. V <sup>-</sup>	
0	0.80	10	780	0.82	11	770	0.81	10	800	0.80	12	790	0.80	12	785
24	0.80	9	770	0.52	8	620	0.43	7	500	0.25	4	280	0.11	1	100

*Effect of pH*

As a preliminary experiment a series of solutions of crystalline pepsin containing different concentrations of hydrochloric acid were kept at 35°C. for 24 hours and the protein nitrogen per ml. of solution precipitated with 2.5 per cent trichloroacetic acid was determined at the beginning and the end of the experiment. The results of this experiment are shown in Table I. The table shows that the more acid the solution the more rapidly the protein is hydrolyzed so that with 2 molar hydrochloric acid only about 10 per cent of the original protein is present after 24 hours. The table also shows that the decrease in

activity is proportional to the decrease in protein nitrogen. The effect of pH on the stability of the protein therefore is similar to that already found for crude pepsin preparations (5).

### *Mechanism of the Reaction*

There are several possible mechanisms which could account for the hydrolysis of the protein under these conditions.

1. The reaction is analogous to ordinary acid hydrolysis of proteins and differs only in that it proceeds more rapidly than is the case with other proteins.

2. The first step in the reaction is the formation of denatured protein which is then hydrolyzed by the acid.

3. The protein digests itself, or denatured protein is formed, by the acid and this denatured protein is then digested by the remaining active protein.

The results as a whole indicate that the reaction is simply a case of ordinary acid hydrolysis and that no denatured protein is formed. The denatured protein is completely insoluble in solutions more acid than pH 3 and if such solutions are heated to boiling rapidly the protein is completely precipitated. No precipitate appears in any of the solutions in the preceding experiment and therefore no appreciable amount of denatured protein is present. In general, there is an optimum pH for the digestion of proteins by pepsin, while the denaturation of proteins increases with the concentration of acid and no optimum is obtained. If, therefore, the first step in the reaction were the formation of denatured protein by the acid and this denatured protein were subsequently hydrolyzed by the active enzyme, it would be expected that in very strong acid solution denatured protein would accumulate in the solution since there should be a point on the acidity curve where the rate of digestion would be decreased by increasing acid while the formation of denatured protein would increase. This is not the case and indicates quite strongly that the observed hydrolysis is not due to enzyme activity. The evidence is not so clear in regard to the possibility that denatured protein is the first step in acid hydrolysis itself. It would be expected that the rate of hydrolysis of denatured protein would increase with the acidity just as does the formation of denatured protein. If it is assumed,

therefore, that the denatured protein is more rapidly hydrolyzed than the native protein, then there would be no accumulation of denatured protein at any degree of acidity and the results would agree with this mechanism. As a matter of fact, if the protein is denatured by boiling and then kept at 50°C. it is found to hydrolyze less rapidly than a similar solution which has not been boiled. The boiled protein, however, is present in the form of a precipitate and it could be supposed that this was the reason for the slow rate of hydrolysis. On the other hand, there is no evidence for the existence of any denatured protein in any of the solutions at any time so that the assumption that the first step in the reaction consists in denaturation is hardly justified. For the time being, therefore, the reaction will be considered simply as a case of ordinary acid hydrolysis.

#### *Decrease in Protein Nitrogen and Activity at pH 1.8*

If any of the products of the hydrolysis of the pepsin were active their presence might be shown by changes in the relative velocity with which the solution hydrolyzes different proteins as well as by a change in the specific activity of the protein in the solution with regard to one substrate. In order to determine whether such changes, either in specific activity or comparative activity with different proteins, occur during the hydrolysis of the pepsin an experiment was carried out at about pH 1.8 and at 50°C. in which the decrease in protein nitrogen and the decrease in activity were determined. The activity was measured by the change in viscosity of gelatin, casein, or edestin, the rennet action on milk, the formation of non-protein nitrogen from casein and edestin, the rate of increase in formol titration of edestin and gelatin, and also the digestion of hemoglobin.

About 2 gm. of four times recrystallized pepsin was stirred into 50 ml. of water and dissolved by the careful addition of 1 ml. M/1 sodium hydroxide. 10 ml. of M/1 hydrochloric acid was then added rapidly and the solution placed at 50°C. 10 ml. samples were withdrawn at various time intervals and 2 ml. M/1 sodium acetate added in order to bring the pH to about 4. The samples were stored in the ice box until the experiment was completed and then analyzed for protein nitrogen and for activity by the various methods mentioned above.

The results of the experiment are shown in Table II. The table shows that the protein nitrogen, as determined by either alkali de-

naturation or precipitation with 2.5 per cent trichloroacetic acid decreases from 1.9 mg. per ml., its original value, to 0.06 while the protein nitrogen, as determined by precipitation with phosphotungstic acid decreases only to about 0.5 mg. per ml. The activity of the solution as measured by any of the methods decreases in almost exact proportion to the decrease in the concentration of protein nitrogen as determined by 2.5 per cent trichloroacetic acid. This fact may be best shown by expressing the results in terms of the specific activity per milligram protein nitrogen. This figure is obtained by dividing the observed activity per ml. of solution by the number of milligrams of protein nitrogen per ml. of solution. The results of the experiment calculated in this way are shown in Table II. The table shows that

TABLE II  
*Hydrolysis of Pepsin at 50°C. and pH 1.8*

Sample	Protein nitrogen per ml.			[P.U.] <sub>mg. P.N.</sub> (Specific activity of protein N)										
	Alk.	2.5 CCl <sub>4</sub> COOH	Ph. T	Gel. V-	Cas. V-	Ed. V-	Cas. V+	Rent.	Cas. S	Ed. S	Cas. F	Ed. F.	Gel. F	Hb
1	1.90	1.80	1.90	13	1000	880	460	73 × 10 <sup>4</sup>	0.47	0.42	0.16	0.08	0.002	0.18
3	0.62	0.60	0.90	13.7	1050	870	350	69 × 10 <sup>4</sup>	0.42	0.39	0.17	0.08		0.17
5	0.20	0.21	0.50	13.0	1020	880	350	71 × 10 <sup>4</sup>	0.46	0.42	0.17	0.085	0.0025	0.18
6	0.06	0.06	0.45	18.0	980	890	420	71 × 10 <sup>5</sup>	0.38	0.46				0.18

the specific activity, as measured by any of the eleven methods used remains constant, within the limits of error of the method, throughout the entire experiment. They prove, therefore, that the activity is entirely due to the original protein remaining in solution and that if any of the split products of the protein possess any activity it must be less than 5 per cent of that possessed by the protein molecule itself. The methods are not sufficiently accurate to detect changes in activity of less than 5 per cent and it is still possible that some of the split products may have a very low activity. In addition to the constant activity the figures also show that there is no change in the relative activity as determined with different proteins. The experiment cannot be continued further since five hundredths of a milligram protein nitrogen per ml. approaches the limit of accuracy of protein nitrogen

determinations although the activity measurements are very much more sensitive. It is necessary, however, to be able to compare the activity with the protein nitrogen content and no information can be obtained from activity measurements made on a solution containing too little protein nitrogen to determine quantitatively.

#### EXPERIMENTAL METHODS

*Activity Measurements* were made as already described (6).

#### *Protein Nitrogen Methods*

*2.5 Per Cent Trichloroacetic Acid.*—1 ml. of solution is added to 10 ml. of 2.5 trichloroacetic acid, heated to 80°C. for 10 minutes, and then cooled. The precipitate is centrifuged, washed three times with 2.5 trichloroacetic acid, dissolved in dilute sodium hydroxide. The solution is washed into the Kjeldahl flask and the total nitrogen determined.

*Alkali Protein.*—2 ml. of the solution is added to 2 ml.  $M/5$  sodium hydroxide, 2 ml.  $N/5$  hydrochloric acid added and then 5 ml. of a solution of  $M/2$  sodium sulfate and  $M/200$  sulfuric acid. The suspension is centrifuged, washed three times with the sodium sulfate solution, the precipitate dissolved in dilute sodium hydroxide, and the total nitrogen content determined by the Kjeldahl method.

*Phosphotungstic Acid.*—Reagent—1.5 gm. of phosphotungstic acid dissolved in 5 ml. concentrated hydrochloric acid, made up to 100 ml. with ethyl alcohol, and then made up to 500 ml. with water. 1 ml. of the solution is added to 5 ml. of this reagent, the suspension centrifuged, washed three times with the reagent, the precipitate dissolved in dilute sodium hydroxide and the total nitrogen content determined by the Kjeldahl method.

#### SUMMARY

The decrease in protein nitrogen and in the activity of solutions of crystalline pepsin at pH 1.8 and 45°C. has been determined. The decrease in activity, as measured with eleven different methods, is in exact proportion to the decrease of protein nitrogen of the solution. The measurements were continued until less than 5 per cent of the original protein remained. These results indicate that none of the split products of the protein molecule possess any appreciable activity compared to that of the original protein.

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# PEPSIN ACTIVITY UNITS AND METHODS FOR DETERMINING PEPTIC ACTIVITY

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Catalysts, by definition, affect only the velocity of a reaction and the activity of a catalyst, therefore, should be expressed as a velocity constant. Since enzymes are generally considered to be catalysts the unit of enzyme activity should also be expressed as a velocity constant. In order to do this, however, it is necessary to know the kinetic equation for the reaction. In the case of most enzyme reactions this equation is not known and it is therefore not possible to express the activity of the enzyme as a velocity constant. This is especially true of pepsin and trypsin. It is necessary, therefore, to use some more or less arbitrary definition of activity and it has been found convenient in the case of pepsin to express the activity in terms of the initial rate of the reaction due to the presence of the enzyme. This definition of activity is useful for analytical purposes and may be used to compare the activity of various quantities of the same enzyme with the same substrate but cannot be used to compare the activity of different enzymes acting on different substrates.

Proteolytic activity, as defined in this way, corresponds to the measurement of optical activity and refers to a property of the solution. The activity caused by unit quantity of a preparation is called the specific activity and in this paper is expressed as units of activity observed per mg. protein-nitrogen added in the enzyme preparation. The specific activity is abbreviated as  $[P.U.]_{mg.N.}^{Prot.}$ . In general the units are similar to those used by Euler (1) and Willstätter (2).

## *Selection of Methods*

The methods described in the present paper have been developed in connection with the study of the purification of pepsin and have been



chosen from a large number of possible methods with regard to their accuracy, convenience, and significance. When a protein solution is acted upon by pepsin a number of chemical and physical changes occur and it cannot be told beforehand whether or not these different changes are due to different enzymes. It is advisable, therefore, to use more than one change in the properties of the protein solution so as to detect the possible presence of more than one enzyme. For the same reason it is advisable to determine the activity with more than one protein. It was found, for instance, in the progress of the purification of pepsin that one fraction was extremely active in the liquefaction of gelatin (3), whereas it was not especially active in changing its chemical properties. The presence of this specific enzyme would not have been suspected had not the determinations been made in several different ways.

The first effect of pepsin on protein solutions is a very rapid decrease in viscosity accompanied by a slower decrease in the quantity of nitrogen present in the form of protein and by a still slower increase in the number of titratable carboxyl (or amino) groups. The optical activity and conductivity also change but the three former properties have been selected as characteristic of the various changes occurring during the digestion of a protein solution.

The method of determining the activity by the change in viscosity has the advantage of rapidity and accuracy (4). Four or five determinations may be completed in less than an hour and comparative determinations may be made with a probable error of less than 5 per cent. When the determination is made at different times with solutions prepared from various samples of proteins the variation is quite marked and may be 100 per cent owing to the difficulty of preparing protein solutions of the same properties at different times with different protein samples. Gelatin preparations are, however, quite reproducible and the change in the viscosity of gelatin has been found to be one of the most accurate, convenient, and reproducible methods. The change in viscosity has the disadvantage that there is some question as to its physical significance and especially as to its relation to the chemical changes (5, 6).

The method of determining the formation of non-protein nitrogen (*i.e.*, nitrogen not precipitated by 10 per cent trichloroacetic acid) is

more troublesome than the viscosity method. It has the further disadvantage that the quantity of non-protein nitrogen produced has no simple relation to the quantity of enzyme except when very small amounts of enzyme are used and under these conditions the determination is not very accurate. Like the viscosity method, the formation of non-protein nitrogen gives results which cannot be directly interpreted in terms of the chemical changes.

The method of determining the digestion by the increase in carboxyl (or amino) groups is much better in this latter respect since the chemical significance of this measurement is clear. The method is not especially accurate or convenient and the titration has to be carried out with considerable care owing to the fact that the increase in carboxyl groups is a very small per cent of the total titration of the solution. As in the case of the formation of soluble nitrogen, the increase in the formol titration is not proportional to the quantity of enzyme except with very small amounts of enzyme which again yield very small and uncertain titrations.

### *Selection of Proteins*

Gelatin, edestin, casein, and a standardized solution of dried milk have been selected for the activity tests since these solutions represent widely different classes of proteins and are the most easily obtainable in preparations of more or less constant properties. In addition to these methods a colorimetric method for determining the formation of soluble tryptophane and tyrosine from hemoglobin has been developed by Anson (7).

### *Activity Units*

An ideal unit of activity should contain factors for all the various conditions affecting the activity of the enzyme so that a constant figure for the activity would be obtained no matter how the determination was carried out. There are so many factors affecting the rate of peptic digestion, however, that it is not possible to collect them all into one constant factor and it is necessary to select arbitrary standard conditions for the determination. In fixing these conditions it is necessary to consider the constancy with which they can be reproduced and the danger of accidental variations, such as changes in the

pH, etc. After these conditions have been selected it is again necessary to make an arbitrary selection of the units in which the activity is to be expressed. Since changes in different properties of the protein solutions are measured by various methods, it is necessary to use different dimensions for the activity units for the separate methods. The following definitions have been selected.

*Unit of Peptic Activity as Defined by Changes in Viscosity of Gelatin, Casein or Edestin Solutions* [P.U.]<sup>Protein V.</sup>—One unit of activity is defined as a change in the specific viscosity of the standard protein solution at 35.5°C. at the rate of 1 per cent per minute, and the quantity of material possessing one unit of activity is that quantity which will cause this rate of change in the viscosity when contained in 5.2 ml. of the standard digestion mixture. This value is determined from the initial slope of the viscosity-time curve as described in detail below. The activity is expressed as per cent change since this value is nearly independent of the protein concentration. The temperature is fixed at 35.5°C. since at this temperature the viscosity of gelatin remains constant with time while at higher temperatures it decreases and at lower temperatures it increases.

*Coagulation of Milk* [P.U.]<sup>Ren.</sup>—In the case of the coagulation of milk the viscosity time curve is not straight but curves upward rapidly so that the initial slope cannot be determined as is the case with the preceding determinations. The percentage increase in the viscosity is therefore determined arbitrarily from the point at which the specific viscosity is doubled and is obtained by dividing 100 by the time in minutes required to cause this change. The physical significance of this figure is evidently the percentage increase in the viscosity per minute which would be obtained if the increase in viscosity with time were linear over this range.

*Activity Units As Determined by the Formation of Soluble Nitrogen* [P.U.]<sup>Prot. S.</sup>—One unit of activity is defined as the liberation of non-protein nitrogen at the rate of 1 milliequivalent per minute at 35.5°C. in 6 ml. of standard digestion mixture, and the quantity of material possessing one unit of activity is that quantity which will cause this rate of change in the formation of non-protein nitrogen when contained in 6.0 ml. of the standard digestion mixture. In this case actual rather than per cent change is used since the former is more nearly indepen-

dent of the protein concentration. The figure may be obtained by interpolating from a series of determinations made with small amounts of enzyme at various times or from the initial slope of a curve obtained by plotting the change caused by increasing quantities of enzyme after the same time interval.

*Activity Units As Defined by the Increase in Formol Titration [P.U.]<sup>Prot. F.</sup>*—One unit of activity is defined as an increase in carboxyl (or amino) groups in 6.0 ml. of the standard digestion mixture at 35.5°C. at the rate of 1 milliequivalent per minute, and the quantity of material possessing one unit of activity is that quantity which will cause this rate of change in the formation of carboxyl groups when contained in 6.0 ml. of the standard digestion mixture. This figure is obtained in the same way as that for the increase in soluble nitrogen. It represents either the increase in carboxyl groups or the increase in amino groups, depending upon whether or not the zwitter ion theory of proteins and amino acids is correct. According to the classical theory it measures the carboxyl (acid) groups.

## EXPERIMENTAL PROCEDURE

### *I. Viscosity*

#### *Standard Protein Solution*

*Gelatin.*—12.5 gm. air-dry isoelectric gelatin prepared as described by Northrop and Kunitz (8) is stirred into 200 ml. of water; the suspension warmed to 45°C. and made up to 500 ml. A few crystals of thymol are added and the solution kept in the ice box. The relative viscosity of this solution should be between 2.1 and 2.3 at 35.5°C. The solution may be kept without change for several weeks at 5°C. but the viscosity decreases slowly at 35.5°C. so that only sufficient of the solution is warmed to 35.5°C. for 1 day's use. Isoelectric gelatin is used rather than acid gelatin since the viscosity of acid gelatin changes quite rapidly. Also the viscosity of isoelectric gelatin solutions is more easily reproducible and less sensitive to slight changes in pH and especially to the addition of small amounts of salts. Very much larger quantities of enzyme than would be required at pH 2 are needed and this also is an advantage since it obviates the necessity of making very high dilutions of the enzyme solution used.

*Casein Solution.*—25 gm. of Kahlbaum's casein (according to Hammersten) are stirred into 500 ml. of water at about 40°C., care being taken to avoid the formation of large lumps. 25 ml. of molar hydrochloric acid are then added slowly and the solution stirred until the casein is all dissolved. The flask is then placed in boiling

water for  $\frac{1}{2}$  hour and then removed and kept in the ice box. The relative viscosity of this solution should be about 4. It varies, however, considerably with different casein preparations and especially with the length and temperature of heating. If the solution is not heated the viscosity is much higher but changes quite rapidly at 35°C. which necessitates a large and uncertain correction. The determinations made with the same casein solution may be reproduced within  $\pm 5$  per cent but different casein solutions prepared from various samples of casein may give values differing 100 per cent, especially in the value for the positive viscosity change.

*Edestin.*—25 gm. of crystalline edestin recrystallized from NaCl at 50°C. are stirred into 500 ml. of water at about 40°C. and 35 ml. M/1 hydrochloric acid added and the solution stirred until the edestin dissolves. The flask is then immersed in boiling water for  $\frac{1}{2}$  hour and the solution kept in the ice box. The relative viscosity of this solution should be about 2.2. It remains constant indefinitely in the ice box and changes only very slightly at 35.5°C. The change in viscosity of this solution is very regular and comparative results are accurate to  $\pm 2$  or 3 per cent when made with the same edestin solution. As in the case of casein, however, the different solutions prepared from different lots of edestin may give quite different results. The viscosity is also sensitive to the length of time and temperature of heating as in the case of casein.

*Milk.*—A number of attempts were made to prepare a reproducible casein solution for the measurement of the rennet activity. The results, however, were extremely variable as were determinations made with different samples of milk. Solutions may be prepared from dried milk powder ("Klim") which are quite reproducible when prepared from the same original sample and which do not vary excessively when prepared from different samples. 50 gm. of the milk powder is stirred into 500 ml. of water at about 40°C. 50 ml. of M/1, pH 5 acetate buffer added, and the solution kept in the ice box until wanted. Great care must be taken to avoid contamination with the slightest amount of active enzyme as exceedingly minute quantities of pepsin will cause very marked changes in the viscosity of this solution. The specific viscosity of the solution should be about 1.7. It may be kept for several weeks in the ice box or for several hours at 35.5°C. without variation.

*Technique of the Determination.*—The methods used for measuring the change in viscosity are the same for all the various solutions and one description will serve for all. Ostwald type viscosimeters are used. If reproducible results with different instruments are to be obtained it is necessary that the time of outflow be long enough to give real viscosity measurements; i.e., at least 20 seconds should be required for the passage of 1 ml. of water. It is convenient to carry out four determinations simultaneously and the subsequent calculations are much simpler if the four viscosimeters used all have the same time for the outflow of water. The ordinary type of viscosimeters supplied by apparatus manufacturers contain 2 ml. with a delivery time of about 70 to 80 seconds. They are clamped vertically in a row in a glass-walled thermostat set at 35.5°C.  $\pm 0.05^\circ\text{C}$ . The pipettes are washed and dried while in position by drawing through water and then acetone. Since the

capillaries are rather fine in these instruments it is necessary to filter all solutions through hard filter paper as any small particles such as shreds of filter paper will clog the capillary. This is the most troublesome source of variation in the measurements. It is also necessary to use accurate stop-watches as measurements are made to 0.2 of a second and irregularities in the curve have frequently been traced to erratic stop-watches.

5 ml. of the protein solution is pipetted into a 50 ml. test-tube and allowed to remain in the water bath until it has reached the temperature of the bath. 0.2 ml. of the solution to be tested is then added to the 5 ml. protein solution and the time of addition noted. The tube is shaken and contents poured into the wide arm of the viscosimeter. The liquid is drawn up into the bulb with gentle suction so as to avoid air bubbles and the time of the outflow measured with a stop-watch. The time at which the viscosimeter is about one-half empty is also noted and this

TABLE I.  
*Calculation of Peptic Activity from Viscosity Time Curves*

Method	Casein V-control	Casein V-	Casein V+	Rennet	Edestin V-	Gelatin V-
$\Delta V$ .....	0.04	0.11	1.20	0.65	0.038	0.024
$V_0$ .....	3.14	3.14	3.14	0.64	1.326	1.05
$\Delta t$ min.....	10	10	10	24	10	10
[P.U.] <sup>Protein V</sup> 5.2 ml. digestion mixture.....	0.13	0.35	3.8	4.17	0.285	0.23
Mg. pepsin N per 5 ml. protein solution.....	0	$2 \times 10^{-4}$	$1 \times 10^{-2}$	$1 \times 10^{-5}$	$4 \times 10^{-4}$	$2 \times 10^{-2}$
[P.U.] <sup>Prot. V</sup> [mg. pepsin N].....		1100	380	420,000	700	11.5
Pepsin unit = [P.U.] <sup>Protein V</sup> = % change in additional viscosity per min. = $\frac{100 \Delta V}{V_0 \Delta T}$						

time is taken as corresponding to that of the viscosity reading. As soon as the liquid has run through the viscosimeter it is drawn up again and another measurement made. In this way a series of times of outflow are determined at intervals of about 5 or 6 minutes. The specific viscosity corresponding to these readings is then calculated by Equation I and these figures are plotted against the elapsed time.

$$[\text{P.U.}]^{\text{Protein V}} = \text{per cent change in viscosity per min.} = \frac{100\Delta V}{V_0\Delta T} = \frac{100(t''_{\infty} - t''_{st})}{(t''_{\infty} - t''_{H_2O})t}$$

$t''_{\infty}$  = outflow time for solution at beginning; this is determined by extrapolating to 0 time;

$t''_{st}$  = outflow time for solution at time  $t$ .

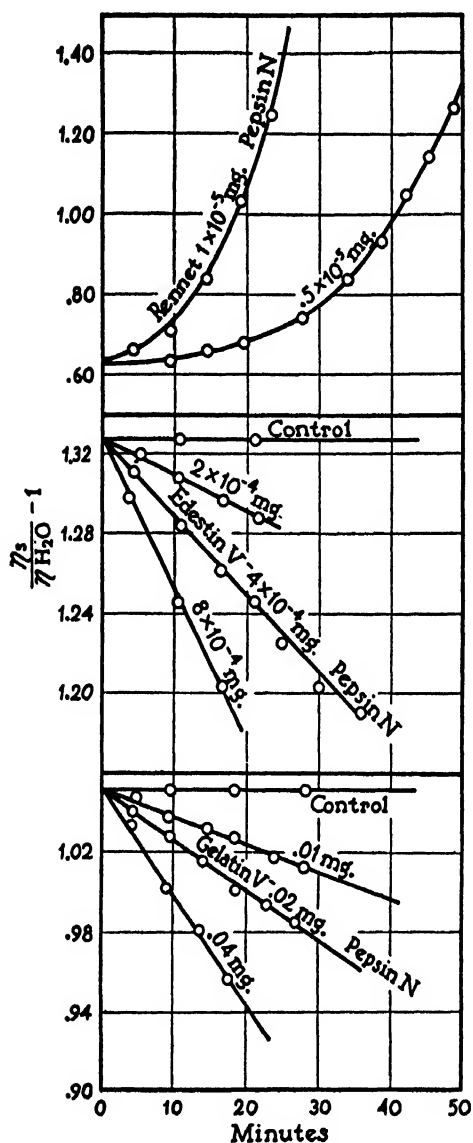


FIG. 1. Change in specific viscosity of milk, edestin, and gelatin solutions with different quantities of crystalline pepsin.

$t''_{H_2O}$  = outflow time for water.

$t$  = time in minutes since enzyme solution is added.

$$\text{Specific viscosity} = V = \frac{t''_s}{t_{H_2O}} - 1$$

An example of the curves obtained in this way with the various proteins are shown

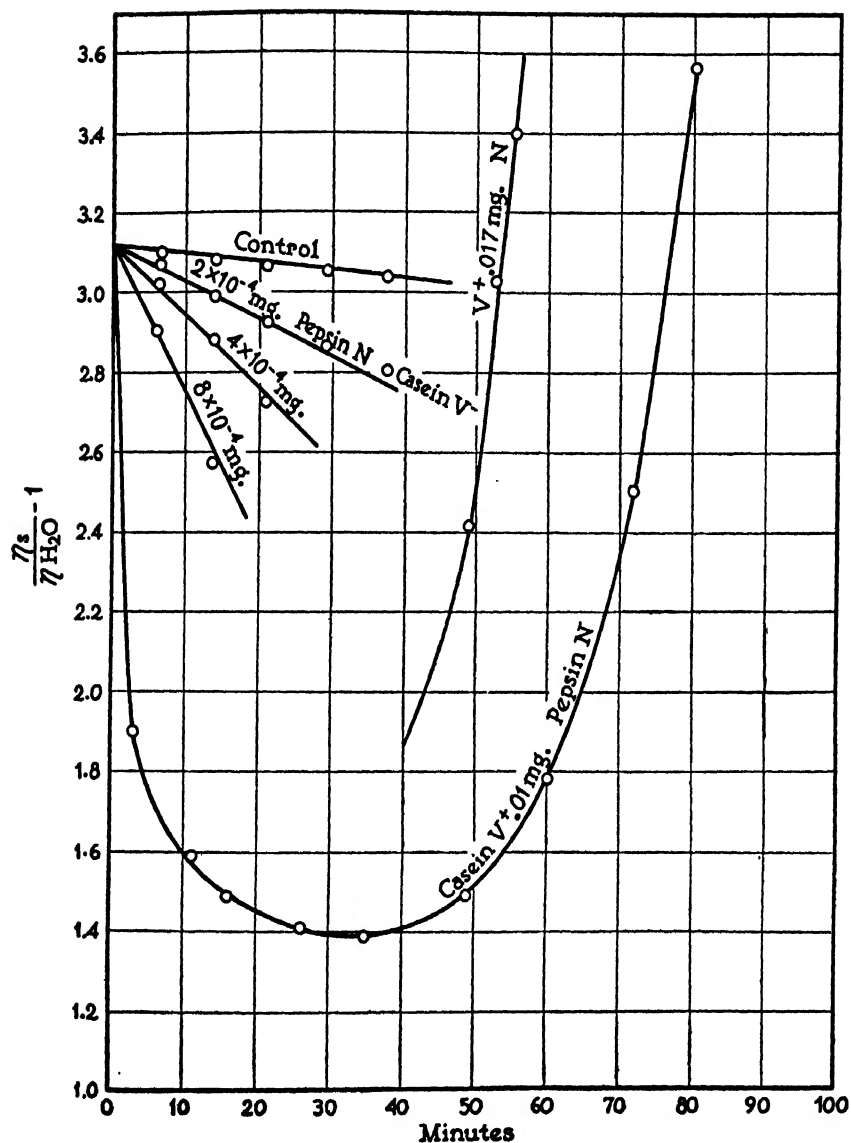


FIG. 2. Changes in viscosity of casein solutions with different quantities of crystalline pepsin.

in Figs. 1 and 2.<sup>1</sup> The number of activity units present are calculated from the slope of these curves as shown in Table I. The calculation has been carried

<sup>1</sup> The curves for the change in viscosity of standard milk previously published (Northrop, J. H., *J. Gen. Physiol.*, 1931, 15, 41) referred to Parke, Davis U.S.P.



out only for one concentration of enzyme but it can be seen from the slopes of the curves that the rate of change of the viscosity is very nearly proportional to the quality of enzyme present.

In calculating the value for the increase of viscosity with casein the slope of the viscosity-time curve is measured at the time when the viscosity has returned to its original value. The time required for the viscosity to return to this point could be used as a measure of the speed as has been done by Holter (9), but in this laboratory this figure appears to vary more with different preparations than does the slope of the line.

## *II. Determination of Activity As Measured by the Production of Non-Protein Nitrogen*

### *Protein Solutions*

This determination is made only with casein and edestin and the same solutions are used as for the viscosity measurements.

In order to obtain a figure for the activity which will be independent of the quantity of the enzyme and of the time of the determination it is necessary to choose conditions under which the rate of the reaction is directly proportional to the time and to the quantity of enzyme. This relation holds only in the very first part of the reaction. It is necessary therefore to work either with very low concentrations of enzyme or very short time intervals. Two general methods are possible. The increase in the formation of soluble nitrogen at different time intervals may be determined and the initial rate of increase interpolated from the curve obtained in this way, or the increase in soluble nitrogen at the end of a constant time interval with varying amounts of enzyme may be determined and the rate of formation of soluble nitrogen with small amounts of enzyme interpolated from the initial slope of this curve. The activity for any one preparation of enzyme, as determined by these two methods, agrees within a rather wide experimental error. It is necessary when testing the activity of an unknown solution to measure more than one concentration of enzyme since in order to be significant the specific activity must be independent of the quantity of enzyme used for the determination and this can only be determined by making the test with more than one quantity. This is especially true of the method by which the change after the constant time interval is used since the curves obtained in this way vary considerably with the purity of the enzyme preparation.

1:10,000 pepsin and not to crystalline pepsin as the legend states. The unit of activity for the rennet action (page 42 of the paper just quoted) is also incorrect and should read "per cent change in additional viscosity of standard milk *per minute*."

### *Technique of the Determination*

*I. Determination from the Initial Rate of Digestion.*—1.0 ml. samples of the pepsin solution are added to a series of tubes containing 5 ml. of the protein solution at 35.5°C. A blank determination must be made with each pepsin solution by adding 1 ml. of a solution of pepsin which has been boiled for 5 minutes. 5 ml. of 20 per cent trichloroacetic acid is then added to the tubes at successive time intervals, the suspensions are filtered, and total nitrogen in 5 ml. of the filter is determined by the micro Kjeldahl method. The quantity of non-protein nitrogen in 6 ml. of the original digestion mixture is calculated from this figure, corrected for the blank determination, and plotted against the time in minutes at which the samples were taken. The activity, by definition, is then determined as the initial slope of these curves and the specific activity of the enzyme sample tested is this slope divided by the quantity of enzyme present. An example of an experiment in which this determination was made with a preparation of crystalline pepsin and with casein

TABLE II

*Calculation of Peptic Activity from Non-Protein Nitrogen Time Curves*

Protein	Casein			Edestin		
Ml. N/50 N per min. (from initial slope).....	0.4	1.1	1.9	0.16	0.34	0.9
Mg. pepsin N.....	0.022	0.05	0.10	0.010	0.020	0.05
Activity per mg. pepsin N. [P.U.] <sub>mg. N</sub> <sup>Prot. N</sup> .....	0.36	0.44	0.38	0.32	0.34	0.36

and edestin is shown in Fig. 3 and the calculation of the activity is given in Table II. The figure for the specific activity obtained in this way is independent of the quantity of the enzyme used, within the error of the method.

*II. Determination of the Activity from the Quantity of Digestion after 1 Hour.*—1.0 ml. of the pepsin solution containing varying amounts of pepsin is added to a series of tubes containing 5.0 ml. of the standard protein solution, and digestion allowed to continue for 1 hour at 35.5°C. The solutions are then analyzed as described for the preceding method and the quantity of soluble nitrogen per 6 ml. of the original digestion mixture plotted against the quantity of pepsin used. The specific activity of the preparation is determined from the initial slope of this curve as in the preceding method. Standard curves, as determined in this way with crystalline pepsin and with casein and edestin, are shown in Fig. 4. The curves for casein and for edestin, with the two particular samples of proteins used in this experiment, are identical. The activity as determined from the initial slope of this curve is about 0.46 milliequivalents per minute per milligram protein nitrogen, which

agrees with the value obtained from the time curves<sup>2</sup> by the preceding methods. In other words, 1 mg. of crystalline pepsin nitrogen has an activity of 0.46 units by this method. In determining the activity of an unknown solution the equivalent number of milligrams of crystalline pepsin nitrogen corresponding to the increase in non-protein nitrogen is read from the curve and this figure times 0.46 gives the number of activity units in 1 ml. of the unknown sample.

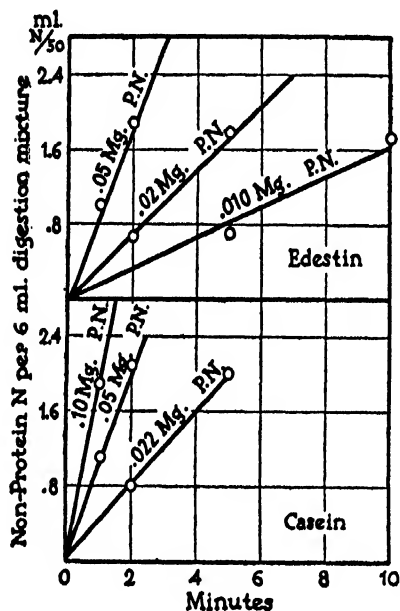


FIG. 3

FIG. 3. Increase in non-protein nitrogen from edestin and casein solutions with different quantities of crystalline pepsin.

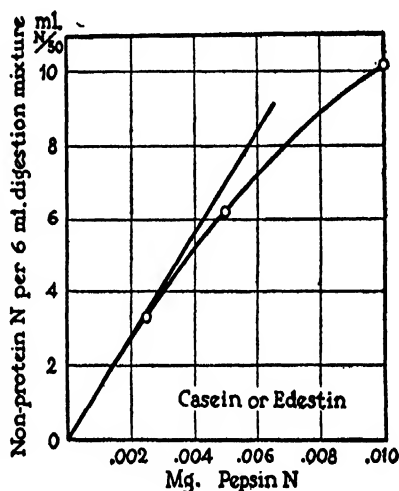


FIG. 4

FIG. 4. Formation of non-protein nitrogen from casein or edestin after 1 hour at 35.5°C. with different quantities of crystalline pepsin.

<sup>2</sup> This experiment is simply a result of the fact that the extent of enzyme reactions in general is determined by the product of the enzyme concentration into the time during which it acts. For instance, if  $E_1$  concentration of enzyme acting for  $T_1$  minutes causes the reaction to proceed  $A$  units, then any other pair of values of  $E$  and  $T$ , such that  $ET = E_1T_1$ , will cause the same amount of reaction,  $A$ . The curves for different quantities of enzyme after the same time, and the curve for the same amount of enzyme at different times could, therefore, be combined into one curve in which the amount of digestion was plotted against the product of the enzyme concentration into the time.

### III. Method of Determination of Activity by the Increase in Formol Titration

#### Protein Solutions

*Gelatin*.—30 gm. air-dry isoelectric gelatin dissolved in about 400 ml. of water at 40°C., 29 ml. of M/1 hydrochloric acid added, and the solution made up to 500 ml. The pH of this solution should be approximately 2.5.

The same edestin and casein solutions were used as for the other methods.

#### Technique of the Determinations

1. *Method of Determining Activity from Time Curves*.—1.0 ml. of the pepsin solution is added to a series of test-tubes each containing 5.0 ml. of the standard protein solution. At various time intervals 5.0 ml. of strong sodium hydroxide is added to the mixture. The strength of the sodium hydroxide is adjusted by trial until 5.0 ml. is just sufficient to bring the solution to a faint pink to phenolphthalein.

TABLE III

*Calculation of Peptic Activity of Crystalline Pepsin by Formation of Non-Protein Nitrogen from Initial Slope of 1 Hour Curves*

Protein	Casein	Edestin
Ml. N/50 N per hr. per 6.0 ml. solution (from initial slope) . . .	7.0	7.0
≈ milliequivalents per min. = [P.U.] <sup>Prot. S</sup> . . . . .	0.0023	0.0023
Mg. pepsin N . . . . .	0.005	0.005
Activity per mg. pepsin N. [P.U.] <sup>Prot. S</sup> <sub>mg. N</sub> . . . . .	0.46	0.46

The alkali should be run carefully down the side of the tube so as not to mix with the protein solution. After the 5 ml. of sodium hydroxide has been added the tube is shaken quickly. This procedure prevents the formation of large clumps of precipitated protein. The tube is then shaken gently until all the protein precipitate is dissolved; 1.0 ml. of 40 per cent formaldehyde solution is then added and the solution titrated to match the standard solution with N/50 sodium hydroxide.

A blank determination is carried out with a sample of the pepsin solution which has been inactivated by boiling.

*Preparation of the Titration Standard (10)*.—The greatest difficulty with the formol titration, as usually carried out, is due to the fact that the color of the solution does not exactly match the standard. This difficulty can be avoided by making the standard out of another sample of the solution itself. To prepare such a standard a sample is run in the same way as for an actual determination. 1 drop of 0.1 per cent phenolphthalein and a large excess of alkali is added so that the indicator develops its maximum color. In titrating the unknown, 1 drop

of 0.5 per cent phenolphthalein is used and the titration continued until the unknown exactly matches the standard. By this method the end-point can usually be determined to less than  $\pm 0.05$  ml.  $N/50$  sodium hydroxide. The difficulty with this method is that the total titration is very large so that if it were carried on entirely with fiftieth normal alkali a large volume of alkali would be required and the end-point would be very uncertain. This difficulty is avoided by using a small volume of concentrated alkali to neutralize most of the acid and

TABLE IV

*Calculation of Peptic Activity from Formol Titration Time Curves*

Protein	Gelatin				Casein				Edestin		
Ml. $N/50$ formol titration per min. ....	0.05	0.075	0.10	0.17	0.07	0.10	0.18	0.40	0.05	0.10	0.25
Mg. pepsin nitrogen. ....	0.60	1.0	2.0	3.0	0.012	0.020	0.04	0.09	0.005	0.011	0.028
Milliequivalents titration per min. per mg. pepsin N [P.U.] <sup>Prot. F</sup> <sub>mg. N</sub> ..	0.0016	0.0015	0.0010	0.0011	0.11	0.10	0.09	0.09	0.20	0.18	0.18

TABLE V

*Calculation of Peptic Activity of Crystalline Pepsin by Formol Titration from Initial Slope of 1 Hour Curves*

Protein	Casein	Edestin	Gelatin
Ml. $N/50$ per hr. per 6.0 ml. digestion mixture (from initial slope) .....	2.2	2.0	0.5
$\infty$ milliequivalents per min. = [P.U.] <sup>Prot. F</sup> ..	0.0007	0.00066	0.00017
Mg. pepsin nitrogen. ....	0.01	0.004	0.2
Activity per mg. pepsin N. [P.U.] <sup>Prot. F</sup> <sub>mg. N</sub> .....	0.07	0.16	0.00085

finishing the titration with dilute alkali. It is necessary to measure the concentrated alkali to 0.01 ml. which can best be done by means of a delivery pipette graduated both above and below the bulb. It is also necessary to measure the 5 ml. of protein solution with the same accuracy since a small error in this volume affects the final titration value very markedly owing to the high acidity of the solution. Owing to changes in the viscosity of the protein solution samples cannot be measured with sufficient accuracy if the digestion is carried on in a large volume and 5.0 ml. samples pipetted out at different times.

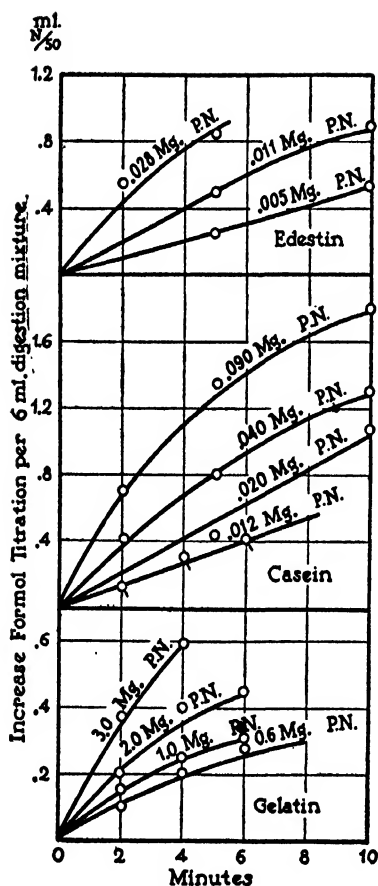


FIG. 5

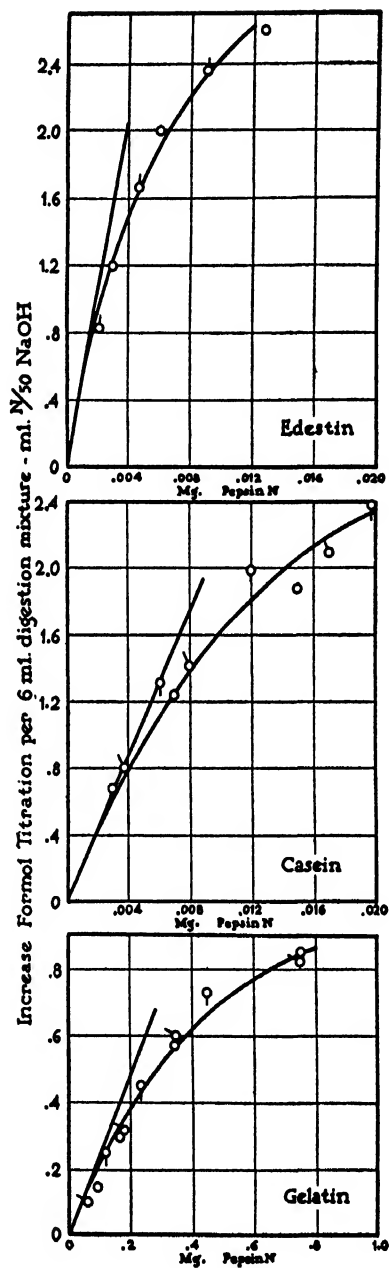


FIG. 6

FIG. 5. Increase in formol titration of gelatin, casein, and edestin with different quantities of crystalline pepsin.

FIG. 6. Increase in the formol titration of gelatin, casein, and edestin after 1 hour with different quantities of crystalline pepsin.



2. *Method of Determining Activity from the Titration Values.*—The increase in the titration per 6.0 ml. of the digestion mixture is plotted against the time of digestion and the activity determined from the initial slope of this curve exactly as in the case of the non-protein nitrogen method. A series of determinations made in this way with crystalline pepsin and with casein, gelatin, and edestin are shown in Fig. 5 and Table IV. The specific activity is independent of the quantity of enzyme within the rather wide error of the method.

3. *Method of Determining the Activity from the Amount of Digestion after 1 Hour at 35.5°C.*—A standard curve for the increase in the formol titration after 1 hour with various quantities of the enzyme is prepared in the same way as for the non-protein nitrogen method. The specific activity is calculated from the initial slope, as described above, and determinations with an unknown sample are made by interpolating from the curve as described for the previous method. The curves obtained with crystalline pepsin and with casein, edestin, and gelatin are shown in Fig. 6 and the calculation of the specific activity in Table V.

### *Accuracy of the Formol and Non-Protein Nitrogen Methods*

In comparative experiments with the same protein preparations the activity of an unknown solution may be determined with an accuracy of about  $\pm 5$  per cent for one determination but different casein and especially different edestin preparations vary, for some unexplained reason, quite considerably. The value obtained with gelatin is much more constant with different gelatin preparations but the experimental error is greater than with casein or edestin owing to the small titration value. A summary of the specific activity of several preparations of crystalline pepsin as determined by these methods is shown in Table VI. The last line of the table gives the concentration of the solution of crystalline pepsin used for the determination. The table shows that the activity of the various preparations as measured by any one method is quite constant except for the casein  $V^+$  method and the casein and edestin formol titration. With these methods the values for the activity found with the last preparation is considerably higher than that found for the first preparation reported. A sample of the first preparation had been preserved in glycerin in the ice box and when measured with the protein solutions used to determine the activity of the last preparation, showed the same activity. The variation, therefore, is due to variation in the protein solutions rather than in the pepsin. The activity units used in this table are the same as those used in the paper on the preparation of gelatinase (3) except that the present ones are expressed as activity per milligram pepsin nitrogen while in the paper referred to the activity was expressed as per gram nitrogen. They differ, however, from the activity units used in the first paper on the activity of crystalline pepsin (11) since in that case the activity, as determined by changes in viscosity, was arbitrarily assumed to be numerically equal to that determined by formol titration.



## SUMMARY

Experimental methods are described for determining the activity of pepsin preparations by means of changes in the viscosity of gelatin, casein, edestin, and powdered milk solutions, and by the rate of formation of non-protein nitrogen from casein and edestin solutions, or by the increase in formol titration of casein, edestin or gelatin.

Activity units for pepsin are defined in terms of these measurements.

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# THE ESTIMATION OF PEPSIN WITH HEMOGLOBIN

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A number of methods have been described for the estimation of peptic activity, many of which are accurate and convenient for comparative experiments but none of which give results which are accurately reproducible with different preparations of the protein used. This variation is due to the difficulty involved in obtaining reproducible protein preparations and keeping them unchanged.

Hemoglobin has been chosen as the protein substrate for the estimation of pepsin because it is easily prepared in large quantities, because it can be stored in solution for a long time without change, because it is rapidly digested, and because the rate at which it is digested by a given pepsin solution does not vary from one hemoglobin preparation to another. Not only is hemoglobin a reproducible protein which can be brought to reproducible conditions but the conditions of digestion have been so chosen that reasonable variations in these conditions have little effect on the amount of digestion.

In general the procedure used to estimate pepsin is this. A pepsin solution is added to acidified hemoglobin. After 5 minutes trichloroacetic acid is added. The resulting precipitate which contains all the pigment and all the undigested hemoglobin is filtered off. The filtrate contains an amount of digested hemoglobin which is a measure of the amount of pepsin used. This digested hemoglobin is estimated by the blue color it gives with the phenol reagent, which reacts with tyrosine, tryptophane and cysteine groups, tyrosine being used as a standard. The intensity of this color is proportional to the amount of enzyme used and to the time of digestion.

*Definition of Activity* [P. U.]<sup>Hb</sup>.—One unit of activity is defined as the formation per minute at 35.5°C. in 6 ml. of the standard digestion

mixture of an amount of digested hemoglobin not precipitable by trichloroacetic acid which gives the same color as 1 milliequivalent of tyrosine, and the quantity of material possessing one unit of activity is that quantity which will cause this rate of change in the formation of digested hemoglobin when contained in 6.0 ml. of the standard digestion mixture (see Northrop, 1932, for a discussion of the definition of pepsin units).

In detail the procedure is this: 5 ml. of a 2 per cent solution of dialyzed ox carbon monoxide hemoglobin in 0.06 N HCl are pipetted into a 175 × 20 mm. test tube and brought to 35.5°C. or 25°C. 1 ml. of enzyme solution is added with an Ostwald pipette, which procedure takes about 4 seconds. The middle of the pipetting period is taken as the time of beginning. To mix the hemoglobin and enzyme solution the test-tube is whirled. After 5 minutes, 10 ml. of 4 per cent trichloroacetic acid are poured into the hemoglobin solution from another test-tube, the suspension is poured back and forth from one test-tube to another, and filtered through fine paper. To 3 ml. of the filtrate in a 50 ml. Erlenmeyer flask are added 20 ml. of water, 1 ml. of 3.85 N NaOH, 1 ml. of the phenol reagent (Folin and Ciocalteu, 1927). Some of this NaOH is to neutralize trichloroacetic acid. The phenol reagent and NaOH are added from automatic pipettes (A. H. Thomas Co.—No. 8210). The standard is made up from 3 ml. 0.1 N HCl containing 0.15 mg. tyrosine.<sup>1</sup> After 5 to 10 minutes, the blue colors are compared, the standard solution being set at 20, and the reading for the unknown solution being called X.

In addition to 0.15 mg. tyrosine, the standard solution contains from the reagents an amount of color-producing substance equivalent to 0.015 mg. tyrosine.<sup>2</sup> The color-producing substance in the 3 ml. of filtrate is therefore equivalent to  $\frac{20}{X}$  (0.15 + 0.015) mg. tyrosine.

Of this 0.015 mg. are due to the color-producing substance in the reagents and 0.01 mg. to the color-producing substance present in the trichloroacetic acid filtrate even when no enzyme is added. The di-

<sup>1</sup> Copper sulfate can be used as a standard instead of the blue solution obtained from tyrosine with the phenol reagent. The colors match if a red color filter is used.

<sup>2</sup> The reagent blank is not measurable. It is deduced from the fact that colors given by tyrosine solutions in the relatively low range of concentrations used are not proportional to the tyrosine concentrations—doubling the tyrosine concentration decreases by some 5 per cent the color given per unit amount of tyrosine. Whether this deduction of a reagent blank equivalent to 0.015 mg. tyrosine is correct or not makes no significant difference to the validity of the calculations which merely provide a correction for the small deviation from proportionality.

gested hemoglobin in the 3 ml. of filtrate is therefore equivalent to  $\frac{20}{X}$  (0.15 + 0.015) - 0.015 - 0.01 mg. tyrosine. This value must be multiplied by  $\frac{16}{3}$  to obtain the digested hemoglobin in the whole 16 ml. of filtrate instead of in the 3 ml. taken for analysis; it must be divided by 5 to obtain the amount of non-precipitable digested hemoglobin produced in 1 minute instead of in 5 minutes; and it must be divided by 181, the molecular weight of tyrosine, to obtain the tyrosine equivalent as milliequivalents instead of as milligrams. The relation between the number of pepsin units in 1 ml. of enzyme solution and the colorimeter reading  $X$  is thus:

$$\text{P. U.} = \left[ \frac{20}{X} (0.15 + 0.015) - 0.015 - 0.01 \right] \frac{16}{3} \times \frac{1}{5} \times \frac{1}{181} = \frac{0.0195}{X} - 0.000147$$

If the digestion is carried out at 25°C. at which temperature it is 1.82 times slower than at 35.5°C. the right hand expression must be multiplied by 1.82 since the unit of activity is defined as the rate of reaction at 35.5°C. By the use of a curve in which P. U. is plotted against  $X$ , one can in practice avoid all calculations.

In the table are recorded actual estimations in terms of pepsin units of the activities of a series of solutions of five times recrystallized pepsin. The constancy of the specific activity, that is the activity per mg. protein nitrogen per ml., is an expression of the fact that the amount of non-precipitable color-forming substance liberated from hemoglobin is proportional to the pepsin concentration.

Protein N per ml. pepsin solution	Activity units [P. U.] <sup>Hb</sup>		Specific activity [P. U.] <sup>Hb</sup> mg. N	
	35.5°C	25°C.	35.5°C.	25°C.
mg.				
0.0180		0.00326		0.181
0.0144		0.00260		0.180
0.0108	0.00194	0.00206	0.180	0.191
0.0072	0.00134	0.00136	0.185	0.189
0.0054	0.00100	0.00103	0.185	0.191
0.0036	0.00065	0.00064	0.180	0.178
0.0018	0.00034		0.189	
			Average . . . . . 0.184	

*The Preparation of Hemoglobin.*—Carbon monoxide is bubbled through whipped ox blood. The blood is centrifuged and the serum and white cells siphoned off. After the corpuscles are washed four times with cold 0.9 per cent sodium chloride, an equal volume of water and a sixth the total volume of toluol are added (Heidelberger, 1922). Carbon monoxide is bubbled through the solution which is then vigorously shaken and allowed to stand in the cold overnight. Next morning the hemoglobin layer is siphoned off, mixed gently with a tenth the volume of centrifuged alumina cream (Tracy and Welker, 1915), centrifuged, and then filtered through coarse paper. Finally the hemoglobin solution is dialyzed overnight in the cold in a shaking dialyzer (Kunitz and Simms, 1928) and stored in the cold under carbon monoxide with toluol as a preservative.

The concentration of hemoglobin is estimated by the Kjeldahl method, the nitrogen content being taken as 17.7 per cent. Hemoglobin is digested with greater difficulty than are the other common proteins. When copper is used as a catalyst, digestion is not complete. When mercury is used, digestion is complete but a preliminary evaporation is necessary to avoid serious foaming. With selenium oxychloride (Lauro, 1931) digestion is rapid and complete and no preliminary evaporation is needed. The concentration of the thrice recrystallized tyrosine is likewise determined by the Kjeldahl method—nitrogen content, 7.74 per cent.

To make up the acid solution of hemoglobin three volumes of 0.1 *N* HCl are added to two volumes of 5 per cent hemoglobin. This acidity (0.06 *N*) is sufficient to give the maximum rate of digestion and is safely on the acid side of the region in which the rate of digestion is sensitive to small changes in the hydrogen ion concentration. Changing the HCl concentration to 0.035 *N* or to 0.08 *N* does not change the rate of digestion more than 5 per cent. Hemoglobin is hydrolyzed very slowly in 0.06 *N* HCl. Acid solutions which have stood in the cold for 10 days have been used for the estimation of pepsin with results the same as those obtained from freshly acidified hemoglobin.

Increasing the hemoglobin concentration from 2 per cent to 3 per cent or decreasing it to 1.5 per cent has no significant effect on the rate of digestion.

Trichloroacetic acid in the concentration chosen (2.5 per cent) gives a precipitate which filters rapidly. If the trichloroacetic acid concentration is decreased 20 per cent, some 10 per cent more color-producing material is left unprecipitated. If the trichloroacetic acid concentration is increased 20 per cent some few per cent less color-producing material is left unprecipitated. 10 per cent trichloroacetic acid, as well as other so called protein precipitants, precipitates a great deal of the digested hemoglobin not precipitated by 2.5 per cent trichloroacetic acid; so that the digested hemoglobin could probably be estimated nephelometrically as well as colorimetrically.

In general, then, the reproducibility of the amount of digestion obtained with a given pepsin solution from different hemoglobin preparations depends essentially on the reproducibility of hemoglobin as a protein rather than on accuracy in fixing

the concentrations of hemoglobin, hydrochloric acid, and trichloroacetic acid, although in practice these concentrations are fixed accurately with little trouble.

The relations between the hemoglobin pepsin unit and other pepsin units are given by Northrop (1932).

A method will be described later for the estimation with hemoglobin of active, native trypsin in the presence of inactive, denatured trypsin, together with a discussion of the peculiar problems involved in the estimation of trypsin, in particular the prevention of the reversal of inactivation and denaturation. By measurements of tryptic digestion, furthermore, one can estimate denatured hemoglobin in the presence of native hemoglobin, for native hemoglobin is not digested by trypsin while denatured hemoglobin is digested at a rate which depends on its concentration.

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# TEMPERATURE CHARACTERISTIC FOR THE ANAEROBIC PRODUCTION OF CO<sub>2</sub> BY GERMINATING SEEDS OF LUPINUS ALBUS

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## I

Temperature characteristics for the consumption of oxygen and for the aerobic production of CO<sub>2</sub> by germinating seeds of *Lupinus albus* were found to be different, and the suggestion was made that the mechanisms underlying the two processes are different but they may act simultaneously (Tang, 1931-32, *a, b*). As a means of confirmation, the differential inhibiting effect of CO was resorted to. It was demonstrated that while the rate of consumption of oxygen can be reversibly inhibited by CO, the production of CO<sub>2</sub> is apparently unaffected by an atmosphere of carbon monoxide (Tang, 1931-32, *d*). It was also pointed out that the rate of anaerobic CO<sub>2</sub> production is of the same order of magnitude as that in air. Although qualitative evidence was lacking regarding the metabolic identity of the CO<sub>2</sub> produced in the two cases, it was considered hardly plausible that the gas produced anaerobically could be quantitatively replaced by the CO<sub>2</sub> given off from an entirely different mechanism when air is made accessible to the respiring material. The purpose of the present paper is to attempt a direct and qualitative identification of the two processes.

The question of the identity of the processes underlying the production of CO<sub>2</sub> aerobically and anaerobically has attracted the attention of many experimenters. Though the evidence is on the whole in favor of identity, direct experimentation has thus far been lacking—at least to the knowledge of the writer. The conclusion has been arrived at mainly from the observations that the rates of production of CO<sub>2</sub> aerobically and anaerobically are affected in the same way by factors such as age, temperature, anesthetics, phosphates, and sugars.<sup>1</sup>

<sup>1</sup> For a review of the subject see: Kostychev, 1927, chapter 2.



A simple and yet direct means of identifying the two processes in an essential respect is afforded by comparing their temperature characteristics—the reverse of the logic used in the previous papers of this series (Tang, 1930–31, 1931–32, *a*, *b*, *d*). For a general discussion of the possible significance of temperature characteristics in biological processes, the reader is referred to the work of Crozier (1924–25, etc.).

## II

The seeds used were in part from the same lot used in the previous experiments, and in part from a fresh lot obtained from the same source upon the exhaustion of the old supply. Preliminary experiments showed that the two lots of seeds behaved alike in relation to temperature and the effect of CO. They were treated in the same manner as described in previous accounts (*e.g.*, Tang, 1930–31).

Modified Warburg microrespirometers designed for the study of seed metabolism (Tang, 1931–32, *c*) were used. The experiments were performed in darkness, except during the readings of the manometers when a very weak electric light was turned on opposite the manometers. The latter were not shaken during the experiment. The vessels, with the seeds mounted in place, were filled with N<sub>2</sub> by passing the gas fairly vigorously (at a pressure of 50 mm. of Brodie solution in the manometers) through them for at least 7 minutes. The N<sub>2</sub> was purified by bubbling it through an absorption tower containing Fieser solution,<sup>2</sup> then through a solution of Pb acetate to remove any H<sub>2</sub>S generated from the latter, dried over concentrated H<sub>2</sub>SO<sub>4</sub>, and finally through the respirometer vessels. When so treated the amount of O<sub>2</sub> in the N<sub>2</sub> was found to be negligible (less than 0.1 per cent when analyzed with a Haldane gas analyzer). 0.1 cc. of water was placed in each vessel to maintain a moist atmosphere.

An experiment was usually started at 18° or 14°C. in a thermostat the temperature of which was kept constant to within 1/100 of a degree. After ½ hour of adaptation and 1 hour of respiration, during which four readings were taken, the temperature was lowered about 2° and the process repeated until the lowest temperature of the range was reached, when the temperature was raised in a manner such that respiration data were obtained at about 1° apart throughout the whole temperature range of the experiment. This is essentially the same procedure used in the previous studies on the temperature characteristics of

<sup>2</sup> The solution is made up of: 13.3 gm. of NaOH, 16 gm. of Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, 4 gm. of Na anthraquinone  $\beta$ -sulfonate for each 100 cc. of H<sub>2</sub>O. See Fieser (1924).

these seeds except that shorter ranges of temperature were employed here. This was unavoidable because, unlike that in air, the rate of production of  $\text{CO}_2$  in  $\text{N}_2$  was found to decrease with time, the more rapidly the higher the temperature. In Fig. 1, the course of  $\text{CO}_2$  production for the seeds at three different temperatures is plotted against time for a period of 12 hours after  $\frac{1}{2}$  hour was allowed for

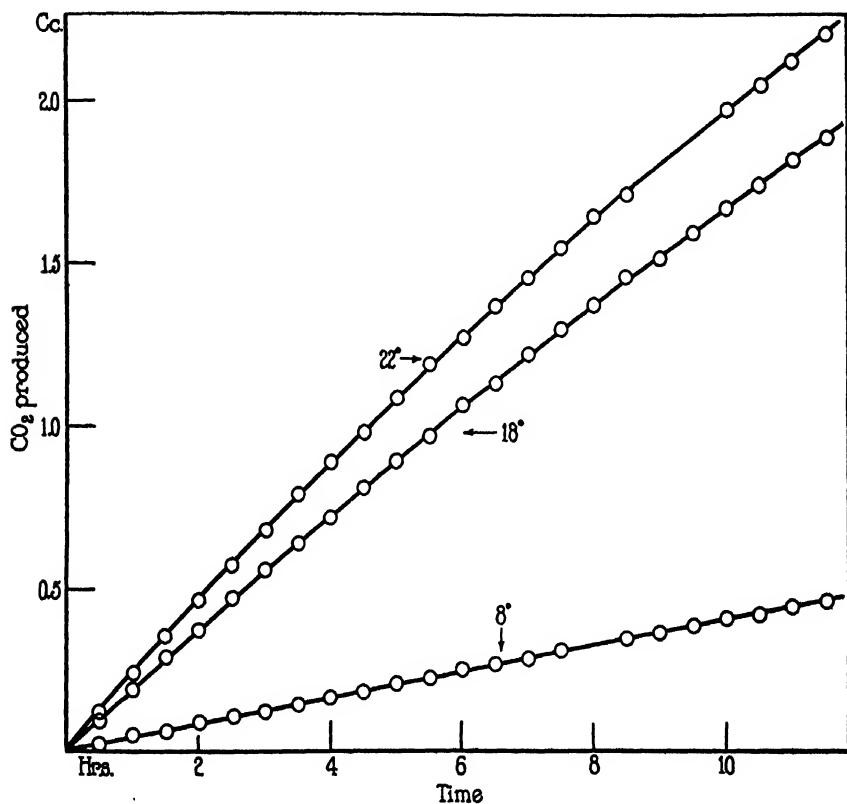


FIG. 1. Course of anaerobic  $\text{CO}_2$  production for germinating seeds of *Lupinus albus* at 8°, 18°, and 22°. Ordinate, cc. of  $\text{CO}_2$  produced; abscissa, time in hours. Readings were taken at  $\frac{1}{2}$  hour intervals after  $\frac{1}{2}$  hour of thermal adaptation in each case. Room temperature, about 24°C.

adaptation in each case (room temperature was  $24 \pm ^\circ\text{C}$ ). These are representative of a large number of experiments. It is noticed that the rate is constant at 8° and almost so at 18°, which are the lower and upper limits of the temperature range used. For practical purposes, and for short durations such as 1 hour at each temperature, the rate

may be treated as being constant at temperatures lying within these limits.<sup>3</sup> This is not the case for higher temperatures, of which the curve for 22° is fairly representative. The assumption that the rate of production of CO<sub>2</sub> between 8° and 18° during the entire period of the

TABLE I

Experiments illustrating the constancy of the rates of CO<sub>2</sub> production (c.mm. CO<sub>2</sub> per seed per hour) during short time intervals at each temperature, and the absence of stimulation when temperature is changed either from a higher to a lower one, or *vice versa*. The data are obtained from two separate series of experiments: one, in which the temperature is changed from 18° to 16°, and another, from 11° to 14°, with 30 minutes for adaptation at each temperature. Because of the small changes on the manometers, the figures are only reliable to  $\pm 7$  per cent.

Experiment No.....		1	2	3
Successive periods of 15 min.				
18°	1	5.8	7.1	5.6
	2	6.5	6.4	4.9
	3	5.8	7.1	5.6
	4	7.1	7.1	4.2
16°	1	4.5	3.6	3.5
	2	6.5	5.7	4.2
	3	4.5	5.0	4.2
	4	5.8	5.0	4.2
11°	1	3.3	4.3	4.1
	2	4.6	6.5	6.9
	3	2.6	5.0	4.8
	4	4.6	5.8	6.2
14°	1	5.2	7.9	9.7
	2	5.9	7.2	6.9
	3	4.6	7.2	8.3
	4	5.2	7.2	7.6

experiment may be treated as being constant with respect to time is justified in the following way. Table I presents two series of experiments, one of which was started at 18° and then the temperature was lowered to 16°. At each temperature  $\frac{1}{2}$  hour was allowed for thermal adaptation, after which readings were taken at 15 minute intervals

<sup>3</sup> See discussions below.

for an hour. The other series was started at  $11^{\circ}$  and then raised to  $14^{\circ}$ , and the readings were taken in the same manner. The figures are constant to within  $\pm 7$  per cent, which is the limit of accuracy of the method *for such small changes* in the manometers. That temperature alterations as practiced here and in the previous experiments did not produce any "stimulating effect"<sup>4</sup> is made evident in the following tests. In previous studies on the temperature characteristics for the oxygen consumption of these seeds, a set of observations were made (but not published) in which the rates of oxygen consumption of the seeds were observed first at  $13^{\circ}$ . After going through the usual temperature changes, the rates were observed again at the same temperature. The rate was 72 c.mm. per seed per hour at  $13^{\circ}$  before and after the change for a typical experiment. During the course of the present investigation, three experiments were performed in the same manner on the production of  $\text{CO}_2$  in  $\text{N}_2$ , with  $14^{\circ}$  as the initial and final temperature. The rates were 28.0, 33.0, and 20.5 c.mm. of  $\text{CO}_2$  produced per seed per hour at the beginning, and 29.5, 33.0, and 21.1 respectively at the end, when the temperature of the experiment was changed successively, about  $2^{\circ}$  at a time, from  $14^{\circ}$  to  $7.5^{\circ}$  and back to  $14^{\circ}$  with a time lapse of about 12 hours. These preliminary experiments clearly indicate that for the study of the rate of respiration of the seeds as a function of temperature, under these conditions, the sudden changes of temperature and lapse of time (at lower temperatures at least) are not complicating factors.

At the end of an experiment, which usually lasted for about 12 hours, the hypocotyls of the seedlings showed little sign of elongation, though they were somewhat larger in diameter than at the beginning. When planted on moist sawdust, the seedlings showed healthy growth in every case.

### III

The results of two series of experiments, each consisting of five individual observations, were brought together by factors and presented graphically in Fig. 2. In the figure, the ordinate represents the logarithms of the relative rates of  $\text{CO}_2$  production; the abscissa,  $1/T$

<sup>4</sup> For discussion of this question see: Palladin (1899), Blanc (1916), and Harrington (1923); also Crozier and Navez (1930-31).

$\times 10^6$  in Kelvin units. Where the number of circles is less than five, some of them overlap. The points fall into a band the slope of which yields  $21,500 \pm$  calories with an extreme variation of  $\pm 10$  per cent.

Fig. 3 gives the results of an experiment, typical of many, where the temperature was raised from  $16^\circ$  to  $22^\circ$  and then lowered to  $16^\circ$ . The order in which the observations were made is indicated by the numbers near the circles. The points did not fall on any straight line in ascending the temperature scale, but in descending from  $22^\circ$  to  $16^\circ$ , they fall

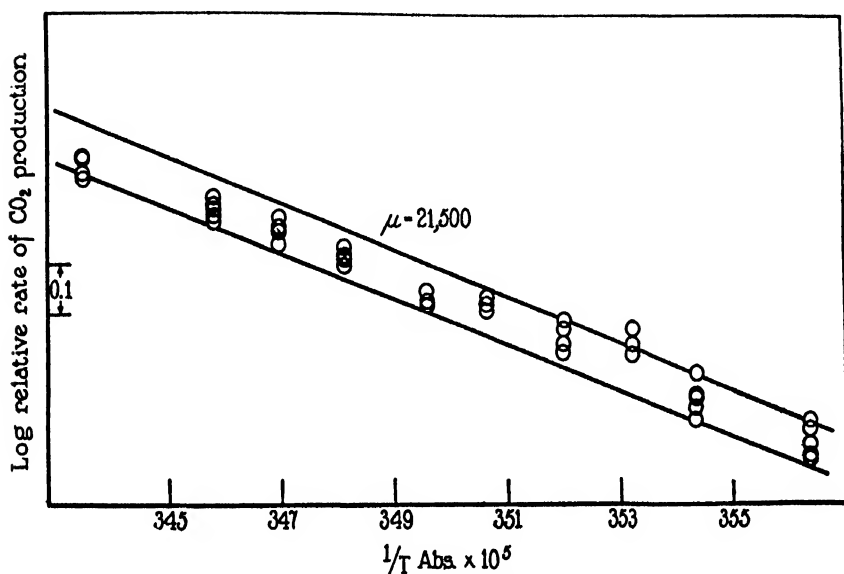


FIG. 2. Log relative rates of CO<sub>2</sub> production by germinating seeds of *Lupinus albus* in nitrogen plotted against  $\frac{1}{T}$  (Kelvin units). Two groups of five experiments each were brought together by factors. Where the number of points is less than five, some of them overlap. The points fall within a band the slope of which yields  $21,500 \pm$  calories.

on a straight line the slope of which yields  $22,000 \pm$  calories. It is recalled that  $19.5^\circ$  was previously found to be the critical temperature both for oxygen consumption and for the production of CO<sub>2</sub> aerobically by these seeds, and it is likely that this is also the temperature here, but not clearly defined. Behavior of this sort in other organisms at and above the critical temperature is not unknown (e.g., Crozier and Stier, 1926-27).

Another rather unusual phenomenon, but related to the foregoing, was observed in a set of experiments of which the one given in Fig. 4 is typical. Two lines with the same slope of  $23,500 \pm$  can be drawn through the points; one through those observed on the downward journey of the respiring material on the temperature scale, and another

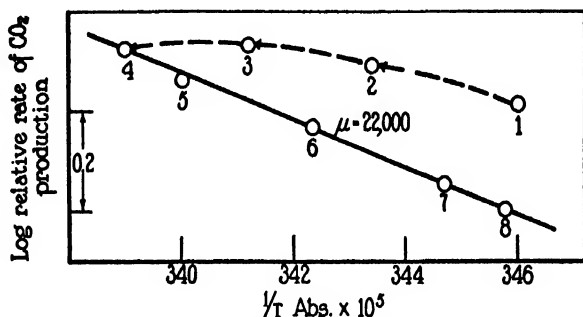


FIG. 3. A typical experiment illustrating the behavior of some of the seeds at temperatures higher than  $18^\circ$ . The points are plotted similar to those in Fig. 2 and the order in which they were obtained is indicated by the numbers attached to the circles.

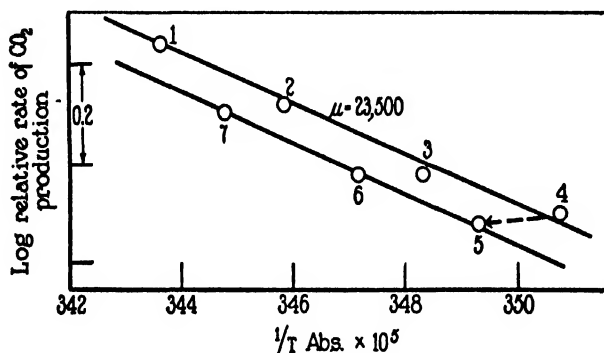


FIG. 4. A typical experiment illustrating the occurrence of the points on two parallel lines yielding the same slope of  $23,500 \pm$  calories, but different in their absolute magnitudes. The points are plotted as in Figs. 2 and 3, with the order in which they were taken indicated by numbers near the circles.

upward, the absolute magnitudes for the latter being smaller. The only statement which we can make at present concerning the meaning of such effects is that phenomena of this nature have been observed in other cases (unpublished observations, Crozier and Stier). Further experimentation is needed to clear up these points.

## IV

The value of  $\mu$  obtained in these experiments, 21,500 (variation 20,000 to 24,000), is somewhat lower than that of  $24,000 \pm$  reported for the same seeds at the same temperature range, but obtained from groups of thirty seeds each, using the Ba(OH)<sub>2</sub> titration method (Tang, 1931-32, *a*). Another value for the same seeds obtained in five experiments performed with the Warburg technic at two temperatures (18° and 8°) only, is  $23,300 \pm$  calories, with an extreme variation in the values of  $\mu$ 's from 22,000 to 25,000 (Tang, 1931-32, *b*). While the mean value of  $23,300 \pm$  is higher than the one reported here ( $21,500 \pm$ ), the ranges of variation overlap. Curiously enough, the value of 21,500 corresponds almost exactly to that for the aerobic production of CO<sub>2</sub> for *Zea mays* (Tang, 1931-32, *a*).

That the value of  $\mu$  as determined in the present experiments is on the whole slightly lower than the ones previously determined for the aerobic production of CO<sub>2</sub> by the same seeds, and that the range of variation is wider, is not at all surprising. At higher temperatures the rates of the anaerobic CO<sub>2</sub> production are only *approximately* constant with respect to time: actually, as has been pointed out, the rates decrease as time goes on. Because of this slight but continuous decrease in the rates, the points at the higher temperatures taken on reascending the temperature scale are lower than what they would have been if the rates were strictly constant. This is clearly revealed by comparing the points at the lower temperatures with those taken at the higher ones in Fig. 2. The former, if treated alone, tend to give a value of  $\mu$  higher than 21,500, and the reverse can be said of the latter; the compromise value of 21,500 calories may certainly be considered to be slightly too low.

In view of what has been said, and also of the fact that the ranges of the individual  $\mu$ 's overlap, we may state that within the limits of error of the experiments, the values of  $\mu$ 's for the production of CO<sub>2</sub> by the germinating seeds of *Lupinus albus*, both aerobically and anaerobically, are found to lie within the limits of 21,000 and 24,000 calories approximately; and that the mechanisms underlying the production of CO<sub>2</sub> by these seeds aerobically and anaerobically may be considered identical in so far as concerns the relation of their rates to temperature.

## SUMMARY

The rate of anaerobic production of  $\text{CO}_2$  by germinating seeds of *Lupinus albus* was studied as a function of temperature between  $7.5^\circ$  and  $18^\circ\text{C}$ . The mean value for the temperature characteristic was found to be  $21,500 \pm$  calories, which is slightly lower than that for the same process under aerobic conditions ( $23,500 \pm$  calories). The values for the individual  $\mu$ 's in the two cases overlap considerably. The possible identity of the processes underlying the production of  $\text{CO}_2$  aerobically and anaerobically is discussed.

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# DARK ADAPTATION AND THE DARK GROWTH RESPONSE OF PHYCOMYCES

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## I

A sporangiophore of the fungus *Phycomyces* elongates at approximately the same rate whether in darkness or in light of several hundred foot-candles intensity, provided it is allowed to become adapted to the particular illumination and if the measurements do not extend over too long a period. When a sporangiophore which is light-adapted and growing at a constant rate is exposed to more intense illumination, a temporary acceleration of growth—the “light growth” response—is produced, followed by a decrease in rate; after a variable interval (usually about 30 minutes) the original rate of growth is regained.

Conversely, when light is suddenly cut off from a light-adapted sporangiophore, a temporary diminution of the rate of growth occurs, followed by a gradual return to approximately the rate of growth in the light. This growth response to sudden darkening has been described for *Phycomyces* by Tollenaar (1923), and named by him the “dark growth” response.

Responses to decreases in visible illumination have been described in the case of a number of higher plants, as the coleoptile of the *Avena* seedling (Tollenaar, 1923), as well as in the shading responses of certain photosensitive invertebrates (*cf.* Crozier, 1915; Hartline, 1925). The same situation is met with in vision, where darkness following illumination may lead to a positive response.

In this paper it is desired to test the idea that the dark growth response of *Phycomyces* represents a decrease in the rate of growth consequent upon the disappearance during dark adaptation of some substance which exerts a continuous influence on the rate of elongation.

## II

The method of the experiment depends on the fact discovered by Tollenaar that the dark growth response of *Phycomyces* appears sooner following darkening the higher the intensity of light to which the fungus was adapted. Actively growing sporangiophores were therefore adapted to a series of intensities covering a wide range, and the reaction times of their responses to sudden complete darkening measured.

Pure cultures of *Phycomyces blakesleanus* ("+" strain) were grown on sterilized flour paste, and placed for experimentation within a plane-walled spectroscopic absorption cell (inside dimensions  $10 \times 10 \times 3$  cm.) provided with a ground glass cover. The atmosphere of the cell was kept saturated by a layer of water on the bottom. Illumination was from above the culture, and was varied by use of a series of concentrated filament, 6 volt tungsten lamps of approximately the same color temperature. Neutral Wratten filters could be interposed, as well as a Petri dish containing a layer of 6 per cent aqueous  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  1 cm. thick, designed to cut out infra-red radiation. This heat screen was particularly important at the higher intensities, but was used throughout the experiments.

The observation chamber was set up, appropriately shielded, in a dark room of relatively constant temperature, the average temperature during the month of experimentation being  $23.0^\circ\text{C}.$ , the extremes a degree above and below. A particular sporangiophore was observed from one side against a faint red light, through a horizontal microscope with an ocular scale. The position of the sporangium on the scale was read off at 12 second intervals and the figures recorded. A plot of these readings against time gives the course of growth before and after the darkening (*cf.* Fig. 1).

The ground glass cover of the observation chamber illuminated the sporangiophore from above with diffuse light coming from a wide angle, and avoided the possibility of the sporangium casting a shadow on the light-sensitive growing zone extending 1 to 2 mm. below it.

In an actual experiment a vigorous sporangiophore was completely adapted to a particular intensity of light by exposure to it for at least 30 minutes. As shown in Fig. 1, readings were taken every 12 seconds for several minutes before the light was put out, and continued for 10 or 15 minutes after that time. When these readings were plotted, the reaction time of the dark growth response was taken as the time from the beginning of the dark period to the first point significantly deviating from the previous rate of growth. This point can be determined objectively by fitting the early, linear part of the curve with

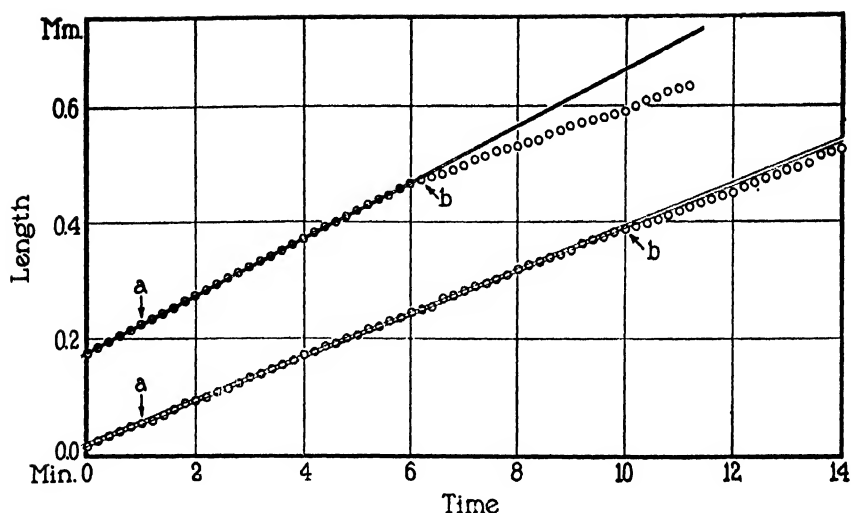


FIG. 1. Dark growth responses following adaptation to 25.4 foot-candles (upper curve), and to 0.00078 foot-candles (lower curve). Complete adaptation to each intensity was secured by at least 30 minutes exposure to light before it was turned off at time *a*. Point *b* is taken as the beginning of response, (*b-a*) as the reaction time. The parallel lines drawn through the linear portion of each curve fit the limits of scatter of the points. The two curves are placed on an arbitrary ordinate scale, the actual length of the sporangiophores at the time of the experiment being several centimeters. Note that at the higher intensity not only is the reaction time shorter, but the *rate* of falling off of the growth rate as well as the apparent size of the depression are greater.

TABLE I

Reaction times of the dark growth response following adaptation to different illuminations; the mean values are averaged from measurements made on a number of different sporangiophores.

<i>I</i>	$\log(I \times 10^4)$	Reaction times	Mean R.T.
<i>foot-candles</i>		<i>min.</i>	<i>min.</i>
334.	6.52	4.6, 5.0, 5.2, 5.2, 5.2, 5.4, 5.4, 5.4, 5.4, 5.6	5.2
25.4	5.40	4.8, 5.2, 5.4, 5.4, 5.4, 5.4, 5.6, 5.6, 5.6, 6.0	5.4
0.435	3.64	5.2, 5.2, 5.4, 5.4, 5.4, 5.6, 5.6, 5.8, 5.8, 6.4	5.6
0.093	2.97	5.6, 6.0, 6.2, 6.2, 6.4, 6.4, 6.4, 6.4, 6.6, 6.8	6.3
0.0096	1.98	6.6, 6.8, 6.8, 7.0, 7.0, 7.2, 7.4, 7.4, 7.4, 7.8	7.1
0.00078	0.89	8.6, 8.8, 8.8, 9.0, 9.0, 9.2, 9.2, 9.8	9.1

two parallel lines drawn at the limits of scatter of the points. The first point falling below this band is taken as a significant deviation.

The figures of Table I are plotted in Fig. 2, where it is seen that the mean reaction time decreases in a regular way as the intensity of the previous adapting illumination increases.  $\log I$  instead of  $I$  is plotted on the abscissa in order to bring out the course of the curve at low

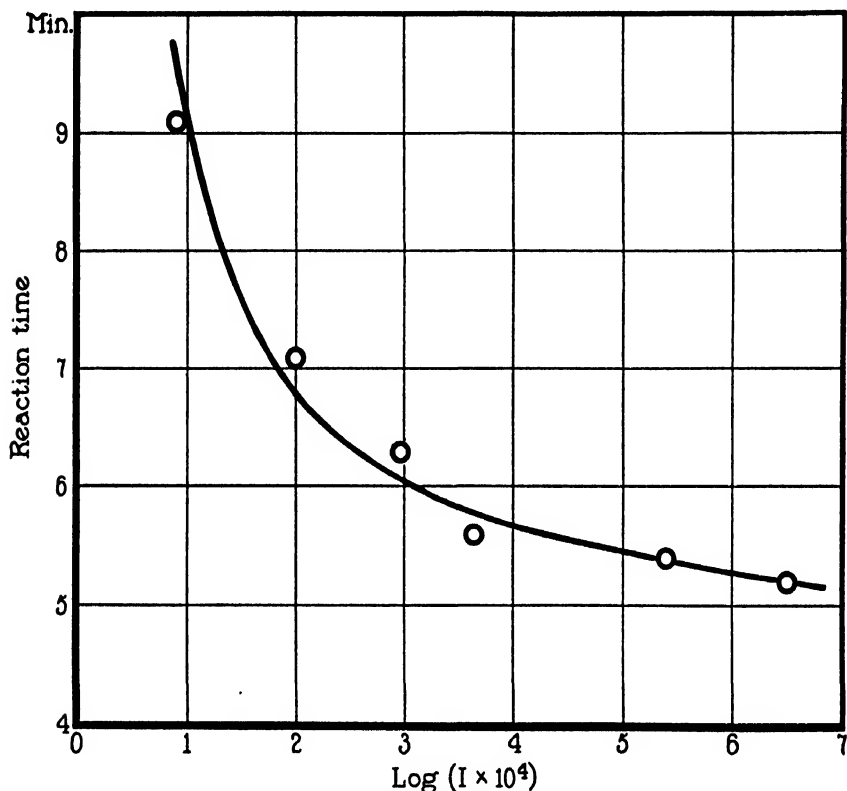


FIG. 2. Mean reaction time of the dark growth response as related to the logarithm of the previous adapting illumination. The curve is the hyperbola

$$R. T. = \frac{1}{0.22 \log (I \times 10^4) + 4.5}$$

intensities. The data of Tollenaar, although few in number, are in general agreement.

### III

Interpretation of these measurements rests on the observation that the reaction time of the dark growth response is compound, consisting

of (1) an *exposure period* during which a certain amount of dark adaptation takes place, and (2) a *latent period* necessary for subsidiary reactions which follow changes in the primary photosensitive system and lead to the growth response. It is a fact that the sporangiophore need not be in darkness during the whole reaction time in order to produce a dark growth response. Temporary darkening for a period much shorter than the total reaction time suffices to call forth a response,

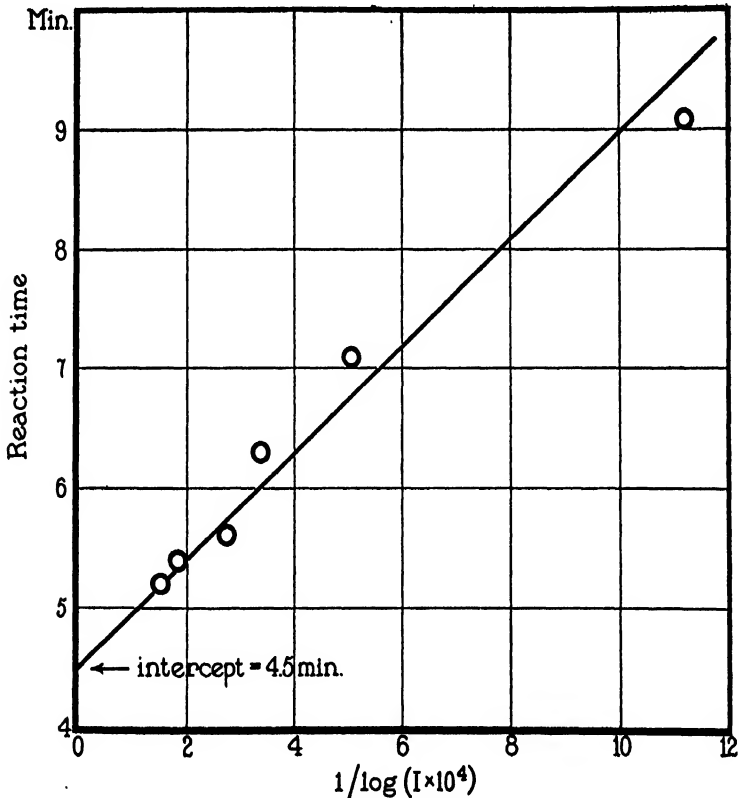


FIG. 3. Reaction time plotted against reciprocal of adapting intensity. The ordinate intercept at  $1/\log(I \times 10^4) = 0$  is the base line of the curve in Fig. 2.

so there is clearly a distinction between the two major components of the reaction time, just as with the light growth response (Castle, 1929–30). The latent period is probably compound and represents the sum of a number of separate latencies. It is taken as constant in duration and independent of the velocity of the primary process of dark adaptation. Since the constancy of this latent period cannot be rigorously

tested by experiment, the only justification of the assumption is that the reaction time measures the time necessary for a minimal effect. Since the initial change necessary to produce this minimal effect is probably constant, the velocities of the subsequent reactions are probably constant.

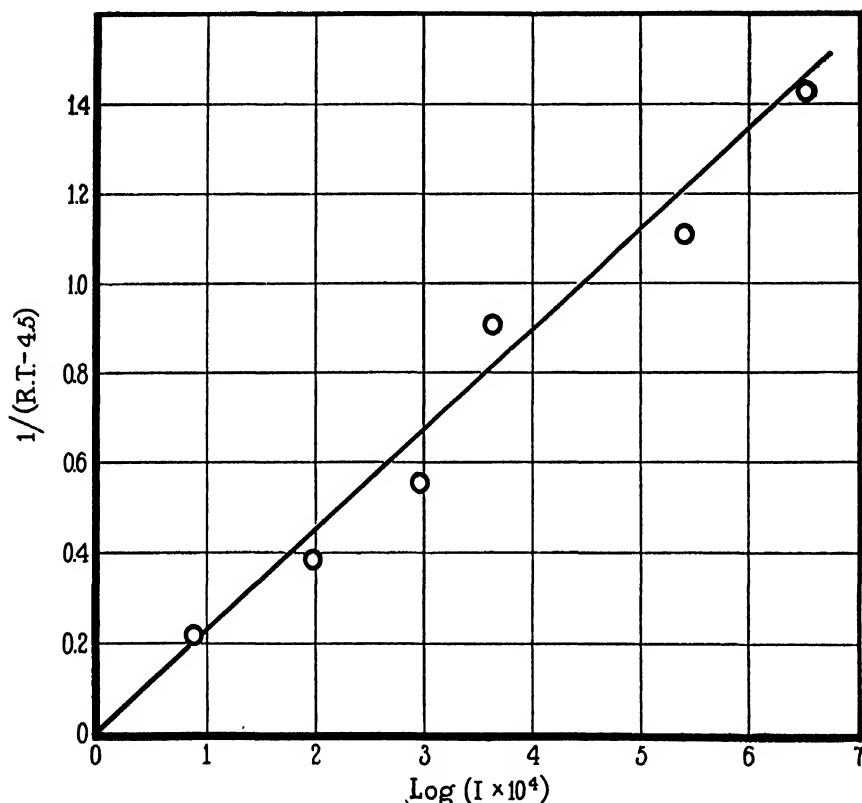


FIG. 4. The inverse plot of the curve in Fig. 3. This shows the rate of dark adaptation during the interval ( $R. T. - 4.5$ ) to be approximately proportional to the logarithm of the previous adapting illumination.

Fig. 2 shows the simple, regular relation which exists between the reaction time and log adapting intensity. What interpretation can be made of this relation? In the first place, the curve seems to descend to a base line representing an  $R.T.$  of 4 to 5 minutes. In a plot of  $R.T.$  against reciprocal of log  $I$ , as in Fig. 3, the points may be said to follow a straight line, their deviations being within the error of the experimental method. Extrapolation to the ordinate intercept gives

the value  $R.T. = 4.5$  minutes, which is the base line to which the hyperbola of Fig. 2 descends at  $\log I = \infty$ , and is assumed to be the actual latent period of the response, that is, the time occupied by secondary reactions which lead to the response.

If  $R.T.$  is a linear function of  $1/\log I$ , conversely  $1/(R.T. - 4.5)$  must be a linear function of  $\log I$ . Fig. 4 shows that this is true within the limits of the experiment. In other words, the velocity of the process which takes place during the interval  $(R.T. - 4.5)$ , measured by the reciprocal of this interval, is directly proportional to the logarithm of the previous adapting illumination. Or, the velocity of dark adaptation is directly proportional to  $\log I$ , where  $I$  is the intensity of the previous adapting light.

TABLE II

Approximate calculation of the amount of change occurring during the exposure time of the dark growth response. Explanation in the text.

$\log(I \times 10^4)$	$R.T. - 4.5$	$\log(I \times 10^4) \times (R.T. - 4.5)$
6.52	0.7	4.56
5.40	0.9	4.86
3.64	1.1	4.01
2.97	1.8	5.34
1.98	2.6	5.14
0.89	4.6	4.10

Since  $\log I$  measures the *rate* of dark adaptation, and since the *time* during which the process must go on in order to lead to response is known  $(R.T. - 4.5)$ , the amount of change necessary to produce the dark growth response can be calculated. Assuming as a first approximation that the rate does not change greatly during the exposure time, the product *rate*  $\times$  *time* should be constant following adaptation to each intensity level. Or,

$$\log I \times (R.T. - 4.5) = \text{constant} \quad (1)$$

The products are given in Table II and are seen to be of the same order of magnitude. This relation immediately derives from the fact that the curve in Fig. 2 is a hyperbola. Its equation may be written as

$$R.T. = \frac{1}{c \log I} + 4.5 \quad (2)$$



where  $c$  is the slope of the line in Fig. 4. By rearrangement,

$$\log I \times (R. T. - 4.5) = \frac{1}{c} = \text{constant} \quad (3)$$

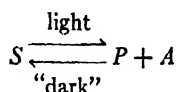
which is identical with (1).

Within the limits of the assumptions made, the constancy of the product *rate*  $\times$  *time* indicates that during the exposure time a constant amount of dark adaptation occurs. Following this, a series of latent period processes is thought to be initiated and to occupy the remaining 4.5 minutes of the reaction time, resulting in the onset of the measured depression of the growth rate.

#### IV

Since the sporangiophores are completely light-adapted before the light is cut off, there may exist at each adaptation intensity a photostationary state in which a particular amount of photosensitive material,  $S$ , is continuously present in equilibrium with its precursors.  $S$  behaves as if it were destroyed by light, reformed in the dark. Kinetically its reformation (dark adaptation) is a process of the second order (Castle, 1928-29).

Hecht (1931; also papers in this *Journal*, 1918, to date) has developed very successfully the idea of a photostationary state to account for photic adaptation and stimulation in the clam *Mya* and the vertebrate eye. His terminology is used in the schema



where  $S$  is the light-sensitive substance, reformed independently of the presence of light by combination of the two substances  $P$  and  $A$ . The simplest treatment of photosensory equilibrium takes the two opposed reactions as truly reversible, but such formulations apply only to the kinetics of the processes.

Changes in sensitivity and the facts of adaptation in the light-sensitive system of *Phycomyces* run surprisingly parallel to those in the case of *Mya* (Castle, 1928-29). The simplest hypothesis to account for the two types of growth response is that some product of

photolysis,  $P$ , exerts a continuous influence on the rate of growth, and is present in greater concentration the greater the light intensity to which the system is adapted. This idea is supported by the typical plots shown in Fig. 1. These show that not only is the reaction time shorter at higher intensities, but the *rate* of falling off of the growth rate as well as the apparent size of the depression are greater.

In these terms, when  $S$  is decomposed by light, more  $P$  is formed, the rate of growth accelerates—the light growth response; when after thorough light adaptation the system is placed in darkness,  $P$  disappears in reforming  $S$ , the rate of growth falls off—the dark growth response. The fact that after complete adaptation either to light or to darkness the rates of growth are approximately equal must find

TABLE III

Calculation of the rate of dark adaptation from equation (4);  $a$  is taken as 100,  $K$  as 1. Explanation in the text.

$\log(I \times 10^4)$	$x$	$x^2$
6.52	80.	6400.
5.40	39.	1521.
3.64	6.3	39.7
2.97	3.0	9.0
1.98	0.97	0.96
0.89	0.28	0.078

its explanation in another limiting factor. This matter will be taken up elsewhere.

It is desired to test the applicability to the case of *Phycomyces* of Hecht's equation for the stationary state

$$KI = \frac{x^2}{a - x}, \quad (4)$$

where  $x$  is the amount of  $P$  (and of  $A$ ) present at equilibrium,  $a$  is the initial amount of  $S$  (taken as 100),  $I$  the light intensity, and  $K$  a constant representing the ratio of the velocity constants of the "light" and "dark" reactions.

Since the "dark" reaction in *Phycomyces* is known to be bimolecular

(Castle, 1928-29), in darkness it will proceed unopposed at a rate dependent on the concentrations of  $P$  and  $A$ . Or, in the dark,

$$\frac{dx}{dt} = -k_2x^2, \quad (5)$$

where  $k_2$  is the velocity constant,  $x$  the amount of  $P$  (and of  $A$ ) present at time  $t$ . The rate of dark adaptation will therefore be proportional to  $x^2$ .

Now Fig. 4 shows that quite apart from any assumption as to the nature of the underlying process, the rate of dark adaptation is proportional to  $\log I$ . Therefore if equation (4) is to apply, values of  $x^2$  calculated from it should also be proportional to  $\log I$ . Inspection of Columns 1 and 3 of Table III shows that this is not the case. The stationary state equation in its simplest form, as in (4), therefore does not fit the facts in the case of *Phycomyces*.

Indeed the only way to get values of  $x^2$  proportional to  $\log I$  out of this type of stationary state equation is to write the partial velocity of the "light" reaction proportional to  $\log I$ , or

$$\frac{\delta x}{\delta t} = k_1 \cdot \log I (a - x) \quad (6)$$

where  $k_1$  is a velocity constant. Under these circumstances, (4) becomes

$$K \cdot \log I = \frac{x^2}{a - x} \quad (7)$$

If  $K$  is small compared with  $a$ , on solving for  $x$  this reduces to

$$x = \sqrt{K \cdot \log I \cdot a}, \text{ or} \\ x^2 = c \cdot \log I \quad (8)$$

where  $c$  is a mixed constant. This approximate solution of (7) states that the rate of dark adaptation, given by  $x^2$ , is proportional to  $\log I$ , which agrees with the relation empirically deduced from the plot of Fig. 4.

Aside from the fact that it yields values of  $x^2$  of the right order of magnitude, no theoretical justification can be given for writing the velocity of the "light" reaction proportional to  $\log I$  instead of to  $I$

directly, as is usual in photochemical reactions. However, it is striking that numerous cases have appeared in biological systems in which the extent or rate of photic action is proportional to  $\log I$ . For example, the velocity of the latent period process in the photo-receptors of *Mya* is proportional to  $\log I$  (Hecht, 1919-20); the velocity constant of the "light" reaction in the photic adaptation of *Agriolimax* is proportional to  $\log I$  (Wolf and Crozier, 1927-28); the magnitude of the electrical response from the retina of *Limulus* is a linear function of  $\log I$  (Hartline, 1929-30). Stehr (1931) has recently found the velocity of the "light" reaction in a number of aquatic arthropods approximately proportional to  $\log I$ . A case from pure chemistry is the linear relation over a considerable intensity range between the darkening of a photographic plate and  $\log I$ . The phase relations in this case are probably no more complex than those prevailing at the receptive zone of *Phycomyces*.

Using equation (7) and taking  $a = 100$ ,  $K = 1$ , values of  $x_1$ , the amount of  $P$  present at each adaptation intensity, have been calculated. These are given in Column 2 of Table IV. When the adapting light is turned out, dark adaptation commences at a rate proportional to  $x_1^2$ , and proceeds for an interval which is known experimentally ( $R.T. - 4.5$ ). At the end of this time sufficient  $P$  has disappeared to affect the latent period processes which lead to the growth response. With the knowledge that the process of dark adaptation is essentially "bimolecular," the amount of change which takes place during the interval ( $R.T. - 4.5$ ) can now be more accurately computed than before, when the rate of dark adaptation was assumed constant during the exposure time. Integration of equation (5), with elimination of the integration constant, gives

$$x_2 = \frac{x_1}{1 - k_2 x_1 (t_1 - t_2)} \quad (9)$$

where  $x_1$  and  $x_2$  are the amounts of  $P$  present at times  $t_1$  and  $t_2$ . In computing with the aid of (9), values of  $x_1$  are taken from Table IV, calculated from equation (7);  $(t_1 - t_2)$  is the period of exposure:  $-(R.T. - 4.5)$ , preceded by the minus sign because  $t_2 > t_1$ ;  $k_2$  has been arbitrarily taken as 0.01. Choice of other equally arbitrary values of  $k_2$  over a large range does not greatly affect the end result, which is

the determination of  $(x_1 - x_2)$ , the amount of  $P$  which disappears during the exposure time.

In Columns 4 and 5 of Table IV are given respectively the values of  $x_2$  and of  $(x_1 - x_2)$ . The constancy of  $(x_1 - x_2)$  is a more delicate test than was carried out above of the idea that during the exposure time a constant amount of dark adaptation takes place. Specifically, a constant amount of  $P$  disappears, and since sensitivity to stimulation by light increases during dark adaptation, is presumed to be converted into  $S$ .

The validity of this test is wholly dependent on the assumption that the light-sensitive system is a closed one, and on the correctness of equations (6) and (7). For the present it seems most useful to take

TABLE IV

Calculation of the critical amount of dark adaptation occurring during the dark growth response after adaptation to different intensities; explanation in the text.

$\log(I \times 10^6)$	$x_1$	$t_1 - t_2$	$x_2$	$x_1 - x_2$
	<i>per cent</i>	<i>min.</i>	<i>per cent</i>	<i>per cent</i>
6.52	22.5	0.7	19.5	3.0
5.40	20.7	0.9	17.5	3.2
3.64	17.3	1.1	14.5	2.8
2.97	15.8	1.8	12.3	3.5
1.98	13.1	2.6	9.8	3.3
0.89	8.99	4.6	6.4	2.6

them as empirically justified. Hartline (1925) in a brief study of the shading response of *Pecten* encountered certain difficulties in the application of equation (4). Possibly a different mode of treatment of the stationary state would obviate the assumption that in the case of *Phycomyces* the velocity of the "light" reaction is proportional to  $\log I$ . It is hoped to test this point directly by a different experimental approach.

# V

It is a striking fact that even with very intense illumination the reaction time of the dark growth response cannot be reduced much below 5 minutes. The theoretical limit, when  $I = \infty$ , has been taken

as 4.5 minutes, all of this interval being taken up by latent period processes. The reaction time of the light growth response, on the other hand, even with stimulation by moderate light intensities, is usually much shorter, and may be as little as 2 minutes.

It is reasonable to regard the light growth and dark growth responses as due to changes similar but of opposite signs in the concentration of some substance which regulates the rate of growth. Therefore the sequence of latent period processes leading to response is the same for each type of response. If this is so, the fact that the latent period is so much longer in the "dark" response than in the "light" response means that the same latent period processes take place at a much greater rate in the latter than in the former. Since the rate of the latent period processes is directly proportional to the rate of photolysis of  $S$  (Castle, 1929-30), it is evident that generally speaking  $P$  is produced in the "light" response at a greater rate than it is removed in the "dark" response. It is usual in biological systems for light adaptation to proceed faster than the reverse process of dark adaptation, due in part to the fact that the velocity of light adaptation is multiplied directly by  $I$  (or some function of  $I$ , as  $\log I$  in the case of *Phycomyces*). The velocity of dark adaptation, however, as shown in equation (5), is determined solely by the concentration of the reactants. The intensity of stimulation resulting from the cutting off of light is therefore dependent only on the amount of decrease in light intensity, and not on "darkness" itself.

#### SUMMARY

Light-adapted sporangiophores of the fungus *Phycomyces* respond to sudden darkening by a temporary decrease in the rate of elongation, after a latent period of several minutes. The reaction time of this "dark growth" response is compound like that of the "light growth" response. It is, moreover, shorter the more intense the previous illumination.

The rate of dark adaptation following adaptation to a very large range of light intensities is found to be proportional to the logarithm of the preceding light intensity. It is shown that a constant amount of dark adaptation takes place before the response occurs.

On the assumption that changes in the rate of growth reflect changes

in the concentration of a substance which at constant light intensity is in equilibrium with a light-sensitive material, possible equations for such a photostationary state are examined. The most reasonable formulation requires that the partial velocity of the "light" reaction be taken proportional to  $\log I$  instead of to  $I$  directly.

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# PULSATION FREQUENCY OF THE ADVISCERAL AND ABVISCERAL HEART BEATS OF *CIONA INTESTINALIS* IN RELATION TO TEMPERATURE

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## I

Pulsation frequency of the heart beat in relation to temperature has been studied in a variety of organisms (*cf.* Crozier, 1924, 1926, *a, b*; Crozier and Federighi, 1925 *a, b*; Crozier and Stier, 1925, *a, b*, 1926, 1927, *a, b*; Fries, 1926; Murray, 1925–26; Glaser, 1929; Parpart and Glaser, 1930). Typical values for temperature characteristics ( $\mu$ ) and critical temperatures were repeatedly found, which fall into definite groups. Of particular interest were cases in which during embryonic development or by way of operations, changes in thermal increments could be observed. Studies on the embryonic heart of *Limulus* (Crozier and Stier, 1927, *b*) showed that more, different  $\mu$  values could be found in earlier stages of development (myogenic pulsation) than later on or in the adult (neurogenic control of pulsation). Similar conditions were met in embryos of *Fundulus* (Glaser, 1929), and in the chick embryo (Parpart and Glaser, 1930). Cases where changes in temperature characteristics were produced subsequent to operative interference are found in *Notonecta* (Crozier and Stier, 1927, *a*) and in *Gonionemus* (Wolf, 1928). Findings of this kind suggest the assumption that during embryonic development or by way of operation the mechanism which controls pulsation has been altered. In organisms where we find different centers which initiate pulsation we might expect to find different controlling mechanisms or pace makers, and an investigation of such cases might throw some light upon the question of the number or diversity of different pace makers in control of pulsation.

In tunicates we meet the fact that the heart beat regularly reverses



after certain intervals, so that the blood is pumped for some time into the region of the gills and afterwards backward into the body region (van Hasselt, 1824, Krukenberg, 1880, Knoll, 1893, Loeb, 1899, Schultze, 1901, Straub, 1901, Hunter, 1902 and 1904, Nicolai, 1908, Hecht, 1917-18, Brücke, 1925, von Skramlik, 1926. In *Ciona intestinalis*, for example, the heart beats regularly in advisceral direction about 140 times, after a few irregular beats the pulsation becomes reversed for about 70 to 100 beats. The total number of pulsations in one or in the other direction is not constant; it differs with age and size of the animal. In general however it can be said that there are always more advisceral pulsations than abvisceral ones. In *Salpa* (von Skramlik, 1926) and other forms the change of direction happens very much more irregularly than in *Ciona*. The circumstances we meet in tunicates suggested a study of the two types of heart beat in relation to temperature.

## II

The material used was collected by the fishing crew of Naples Zoological Station at Mergellina and Capo Posilipo during September, 1929. Usually the animals were kept in running sea water overnight before being used for experimentation. Brought in from the Gulf of Naples the tunica is in most cases covered so heavily with dirt, and the intestinal tract is so little transparent, that it is not possible to see the heart without cutting the tunica. To avoid any kind of an operation which might affect internal pressure and thus influence the heart beat, as described by Hecht (1917-18), it was only necessary to leave the animals for several hours in clean water, where they become more transparent so that one can see the heart quite easily through the tunica by placing the animal so that the anal opening shows to the right hand of the observer. When the heart was easily visible the animals were taken for observation. Less attention was paid to the size of individuals, but in general only smaller animals 6 to 12 cm. in length when fully expanded were used. Stop-watch readings of the time for ten pulsations of the heart in advisceral direction as well as in abvisceral direction were taken, and the results thus obtained were treated separately. Altogether twenty-two animals were used. Over 3000 stop-watch readings were taken for ten pulsations each; in the graphs there are plotted 300 points representing averages of these readings.

During experimentation the animals were placed in dishes about 8 cm. in height, submerged in a large glass aquarium in which the temperature could be changed by adding ice or hot water. The temperature was kept constant by a stirring motor during the run of an observation for about 15 to 20 minutes, to within about 0.3°C. Between the readings at one temperature and another,

when the change of temperature took place, no readings were taken for at least 20 to 25 minutes, to give the animal time enough for thermal adaptation. All the animals were placed in their containers in the same manner. If by contraction of one of the syphons an individual changed his position by rolling over so that the heart was no longer visible, it was brought back into its original position by touching it gently with a thin glass rod. The beating heart was observed by a low power Zeiss binocular microscope.

The successions of pulsation in advisceral and abvisceral directions are somewhat different, the total number of advisceral pulsations being generally greater than the number in abvisceral direction. Similar conditions have been described in earlier papers as cited. In general the sequence of beats is very regular at a given temperature except for the first few and the last few beats before the reversal of direction of the pulsation. The first few beats always follow one another faster and the last beats slower and more irregularly. In the absolute frequency no significant difference could be found between ad- and abvisceral pulsations at any temperature. This fact contradicts somewhat the statement of Roule (1884), who claims to have found a higher pulsation frequency for advisceral beats in younger forms of *Ciona intestinalis*. The results obtained for advisceral pulsations are plotted with the ones for abvisceral pulsations in the same figures. The open marks give the values for the advisceral, the solid marks for the abvisceral pulsations.

### III

The data obtained from twenty-two animals used in these observations are given in Figs. 1 to 3, where the logarithm of the frequency of the heart beat has been plotted against the reciprocal of the absolute temperature. Each point represents an average value of ten readings taken at the corresponding temperature during a run. The different series describe narrow ribbons through which straight lines can be drawn. The relation of pulsation frequency to temperature adheres to the Arrhenius equation  $\frac{k_1}{k_2} = \exp. \frac{\mu}{R} \left( \frac{1}{T_1} - \frac{1}{T_2} \right)$ , where  $k_1$  and  $k_2$  are proportional to velocity constants at the respective temperatures  $T_1$  and  $T_2$ ,  $R$  is the gas constant, and  $\mu$  is the "critical thermal increment" or temperature characteristic.

The data obtained for each animal were at first plotted separately and the values for the temperature characteristics calculated. According to the values found and to the location of changes in the curve of pulsation frequency, the animals were brought into different groups. The rate of pulsation is not always the same in different animals at the same temperature, so that in bringing the animals of one group

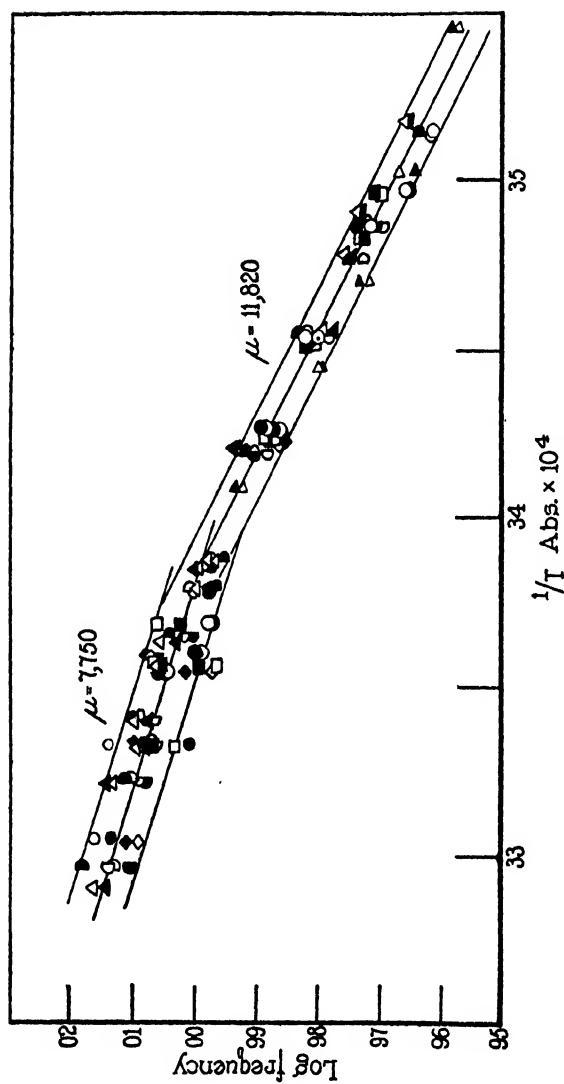


FIG. 1. Observations on pulsation frequency of the heart of *Ciona*. Data from eight animals.  $\mu = 7750$  calories above  $20^\circ\text{C}$ . and  $\mu = 11,820 \pm$  below  $20^\circ\text{C}$ . The readings for advisceral beats are indicated by white marks, for abvisceral beats by black marks.

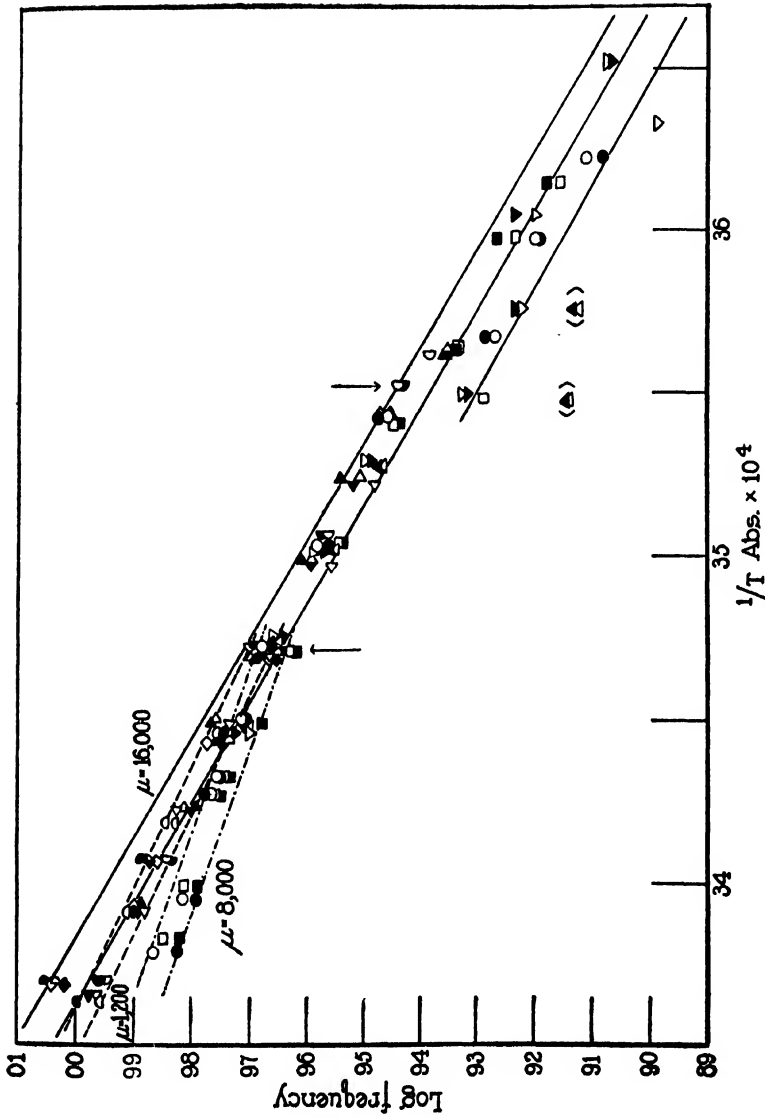


FIG. 2. Observations on pulsation frequency of the heart of *Ciona*. Data from thirteen animals.  $\mu = 16,000$  calories below  $15^\circ\text{C}$ . Above  $15^\circ\text{C}$ . the values are different. One group showing  $\mu = 16,000$ , the second  $\mu = 12,000$ , and the third  $\mu = 8,000$ . The readings for advisceral beats are indicated by white marks, for advisceral beats by black marks.

together the frequency had to be multiplied by a certain factor in each case. It has to be said, however, that in no case where a "break" occurs have the two parts of the plot above and below the critical temperature been multiplied by different factors. Thus the mean values for the temperature characteristics could more easily be determined, and in case a "break" occurred its location was more readily found.

The first group contains observations made on eight animals between 8° and 32°C. At higher temperatures a  $\mu$  value of  $7750 \pm$

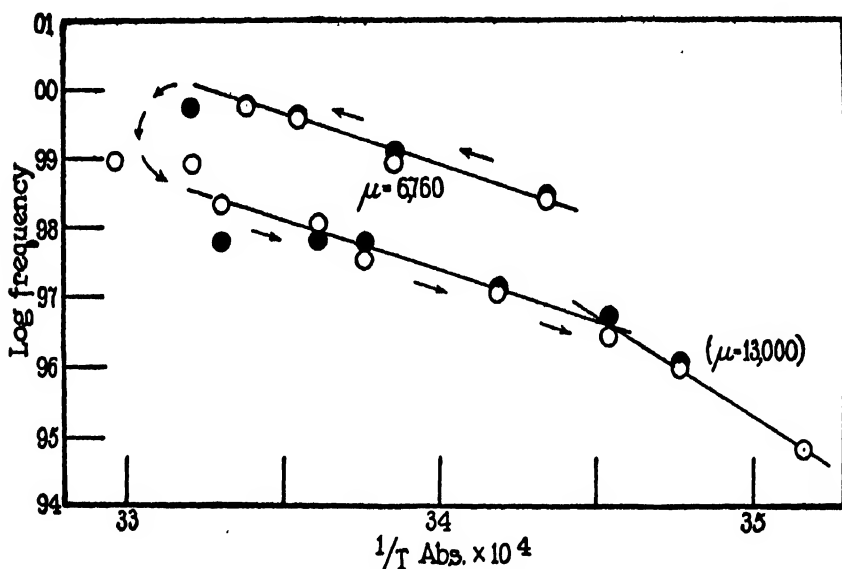


FIG. 3. Observations on pulsation frequency of the heart of *Ciona*. Data from one animal. Above 15°C.  $\mu = 6760$  calories, below 15°  $\mu = 13,000$ , a change of absolute frequency occurs at high temperatures without changing the slope of the line.

calories was obtained. At a temperature of about 21°C. a break occurs, and below this temperature the  $\mu$  value is  $11,820 \pm$  calories. These values fit the data both for pulsation in advisceral and in abvisceral direction (Fig. 1).

The results from 13 other animals investigated are represented in Fig. 2. For the temperature range below 15°C., for all these individuals, we find the temperature characteristic 16,000 calories. Above 15°C. the individuals behave differently. In some of the cases the

$\mu$  value is 16,000 calories just as below 15°C., no change in the curve of pulsation frequency being noticeable. For three animals we find a break at 15°C., with the  $\mu$  value about 12,000 above the critical temperature. In four other animals the break occurs at the same temperature (15°) but the  $\mu$  value found above 15° is 8000. For these four animals there is in addition quite clear evidence of a change of latitude of variation at about 10°C. with a downward shift; the thermal increment, however, stays unchanged.

One additional animal cannot be brought into any of the preceding groups. The  $\mu$  value found for the temperature range below 15°C. is 13,000 calories, whereas above that critical temperature we obtain 6,760. During the change of temperature going up and down the temperature scale we find a difference in the absolute pulsation frequency. The thermal increment, however, is the same in both cases. The reasons for the change in frequency must be found in some destructive changes at the highest temperatures to which the animal had been exposed (above 30°). Similar cases have been recorded in *Notonecta* (Crozier and Stier, 1927, *a*; and Stier, unpublished data).

In most of the cases great difficulty was met in taking readings above 32°C. and below 12°C. Usually the heart stops beating in diastole at temperatures below 10° or 11°C. and at temperatures higher than 31° the pulsation becomes so irregular that no constant readings for pulsation can any longer be obtained. On raising the temperature even higher, the heart stops in diastole until the temperature has been decreased to about 30°C. But even then it is found that in a great many cases the irregular beat does not cease, or that the heart does not come back to function at all for several hours. Similar cases have been described in other tunicates by Hecht, Hunter, Knoll, Wagner, Lingel, Nicolai, Roule. Apparently these extreme temperatures fall too far outside the normal environmental temperature of *Ciona intestinalis* found in the Gulf of Naples, where temperature of the water stays around  $18 \pm ^\circ\text{C}$ . throughout the year.

By examination of the data for each individual a variety of  $\mu$  values could be found. In Fig. 2 for example, we have in all animals the same increment for the lower temperature range but above the critical temperature the  $\mu$  values are quite different. If all the data given were plotted *en masse* without determining the  $\mu$ 's singly a curvilinear

plot would be obtained for which no definite  $\mu$  could be determined (*cf.* Cole, 1929). This would be due to the different temperature characteristics over the upper range of temperatures (*cf.* Crozier, 1924; Crozier and Stier, 1925, *a*; Glaser, 1924).

An attempt was made to use earlier data on pulsation frequency of heart beat in tunicates in relation to temperature. Hecht (1918) has given some data for ten pulsations in ad- and abvisceral direction for *Ascidia atra* at temperatures between 17° and 37°C. The data are relatively few. The approximate  $\mu$  value for the range between 21° and 35°C. is about 12,500+ calories, a value which can be compared easily with values found in *Ciona* in some combination with other magnitudes. In the case of Hecht's observations the temperature characteristic for pulsation in the two directions is the same.

More recently data on *Salpa africana* were published by von Skramlik (1926). In his experiments only readings for pulsation frequencies at temperatures 10° apart were taken for single animals, from which he calculated  $Q_{10} = 2.1$  according to the van't Hoff rule. Computing from his data the time for ten pulsations and plotting the values obtained *en masse* for determination of the temperature characteristic, no definite  $\mu$  value could be determined as the points are too scattered and too few. Furthermore, there might be assumed a change in thermal increment at some critical temperature, just as has been found in *Ciona*, so that a determination of pulsation frequency at only two different temperatures would give us no information at all as to the kind and variety of factors involved in the mechanism controlling pulsation frequency. One fact, however, can be collected from von Skramlik's data, namely that in plotting the frequencies for advisceral pulsations at different temperatures against the abvisceral ones we find an essentially rectilinear relationship with a slope of 1,—which indicates that in *Salpa africana* for both ad- and abvisceral pulsations the frequencies are the same.

#### IV

The  $\mu$  values found for advisceral and abvisceral pulsation frequency of the heart of *Ciona intestinalis* are as follows: 8000, 12,000, and 16,000 in several combinations, and with diverse critical temperatures at 10°, 15°, and 20°C. The fact that the two ends of the heart behave alike in any one individual tested is suggestive for the notion that the same metabolism prevails in the two pace makers at the opposite ends, as is evident also in their identical rates.<sup>1</sup>

<sup>1</sup> The results of these experiments where the pulsation frequency of ad- and abvisceral heart beat is the same at any temperature in any one individual suggests the desirability of testing a possible relationship between the duration of sequences of ad- and abvisceral heart beat to temperature. For these experiments no particular attention was paid to this possibility.

In this connection it might perhaps be pointed out that according to the results obtained in these experiments we are not permitted to draw any further conclusions about the mechanism controlling pulsation frequency. Known facts are that values like 8000, 16,000, and 22,000 are connected with mechanisms at least partly respiratory in type;  $\mu$  values of 10,000 and 12,000 calories have been associated with neuromuscular activities (Crozier, 1924; Crozier and Stier, 1925, *a, b*, 1927, *a*; Wolf, 1928; Steiner, 1932). In experiments with embryonic hearts, where in earlier stages less uniform and more different  $\mu$  values are found than in adult organisms (Crozier and Stier, 1926-27; Glaser, 1929; Parpart and Glaser, 1930), it is tempting to assume that the changes in temperature characteristics are connected with the development of the nervous control of the heart beat. All one can say, however, is that the occurrence of different temperature characteristics for the two cases, embryonic heart and adult, is probably due to the fact that the respective controlling processes are unlike. Only in case one could obtain embryonic preparations of the same state of development but differing concerning the nervous control and then compare their temperature characteristics, might the data perhaps be used for further conclusions (*cf.* Crozier and Stier, 1927, *b*).

## V

## SUMMARY

The frequency of pulsation of the heart of *Ciona intestinalis* increases with temperature in both advisceral and abvisceral direction, according to the Arrhenius equation. The increase in pulsation is the same in both directions. The following  $\mu$  values were obtained: 8,000—, 12,000+, 16,000, in several combinations, with critical temperatures at 10°, 15°, and 20°C. The values found are comparable with earlier findings for activity of the heart in different animals. This quantitative correspondence suggests anew the conception that temperature characteristics may be employed for recognition of controlling processes. The fact that the  $\mu$ 's and the critical temperature are the same for advisceral and abvisceral beats, indicates that the general metabolic condition of the two ends of the heart is the same in any one individual.



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# THE MECHANISMS OF TROPISTIC REACTIONS AND THE STRYCHNINE EFFECT IN DAPHNIA

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## I

Some knowledge of the nature of tropistic reactions has been gained through the investigation of their reversal by various agents (Ewald, 1912, 1914; von Frisch and Kupelwieser, 1913; Loeb, 1918; Crozier and Arey, 1918; Mainx, 1929; Welsh, 1930; Clarke, 1930, 1932). Clarke found for *Daphnia* that geotropism and phototropism could be temporarily reversed by abruptly changing the intensity of light falling upon the animal. A tentative explanation of the reactions of *Daphnia* to light was proposed by postulating the involvement of a reversible photochemical process in the photoreceptors (*cf.* Hecht, 1929, 1931). This interpretation of the responses of *Daphnia* to photic stimulation largely depends, however, upon two assumptions.

First, it was assumed that in *Daphnia* the mechanism for orientation is distinct from that underlying the other aspects of phototropism investigated (Clarke, 1932). Such an assumption was made by Ewald (1914) for the interpretation of his results obtained by stimulating *Daphnia* with intermittent and with colored light. Moreover, the assumption is strongly supported by the fact that changes in illumination which reverse the sign of phototropism do not also reverse the orientation of the body: the animal tends to maintain a position with its dorsum toward the light source under all circumstances (Fig. 1).

Secondly, it was assumed that a change of sign of phototropism involves the control of antagonistic muscles in the swimming appendages which are reciprocally innervated. It is conceivable that the postural angle of the antennae which are chiefly responsible for locomotion is controlled directly by the impulses arising in the photoreceptors. The result would be that, following a change in light

intensity, the antennae would be forced by changes in muscular tensions into such a position that subsequent swimming movements would carry the animal toward the light. Observation, however, does not tend to support a strict interpretation of this view, if it involves the proposed mechanism alone. For, under these circumstances *Daphnia* would never move in a positive direction except when the tilted backwards position for swimming was assumed. It has been reported that *Daphnia* suddenly made photopositive *sometimes* turn around and swim frontwards, scuttle from side to side in a zig-zag

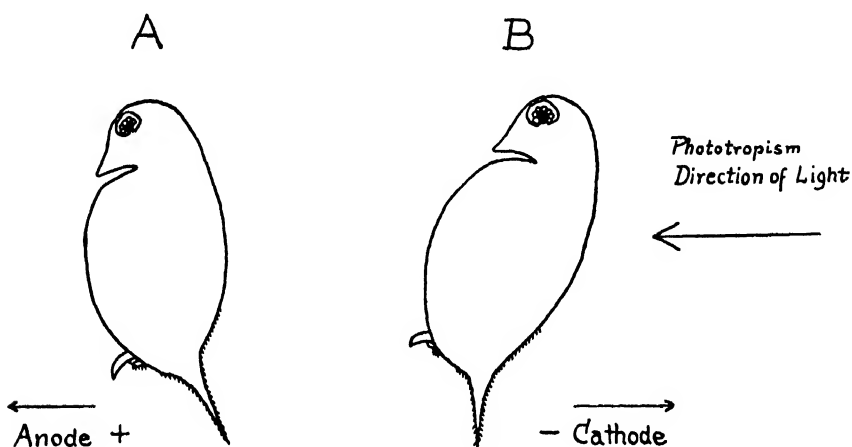


FIG. 1. Diagram to illustrate the usual position of *Daphnia* (A) when swimming "forward" and (B) when swimming "backward." (A) represents an individual which is negatively phototropic or anodically galvanotropic, (B) represents an individual which is positively phototropic or cathodically galvanotropic. Note that in both cases the organism "faces" in the same direction. Antennae omitted.

course, or even bump along the bottom head down (Ewald, 1914; Clarke, 1932).

Accordingly, we are not in a position at present to decide whether stimuli from the photoreceptors produce directly a change in position of the antennae which automatically results in a characteristic phototropic movement, or whether the stimulus arouses a general tendency to move toward or away from the light which secondarily calls forth suitable swimming movements. However, we do know that *Daphnia* swims tilted forward when it is photonegative and usually swims tilted backward when it is photopositive. This difference in position could

be accounted for by the presence of antagonistic muscles in the antennae controlled by reciprocal innervation. A stimulus from the photoreceptors evoking the forward position of the antennae would result in negative phototropism, and a stimulus producing the opposite position of the antennae would result in a tilting of the animal's body backward and a photopositive swimming movement.

Since the action of strychnine has been used as a test of the presence of reciprocal innervation in reflexes (*cf.* Knowlton and Moore, 1917; Moore, 1918-19, 1923-24; Crozier, 1919-20, 1927-28; Crozier and Federighi, 1924-25; Fries, 1927-28) it was proposed to use this method in studying the responses of *Daphnia* to light and to galvanic current. Modifications of tropistic reactions following strychninization should serve as a test for the assumptions under consideration.

## II

To test the effect of strychnine upon the phototropism of *Daphnia*, single animals were placed in a glass trough (8 cm. long, 3.5 cm. wide, and 2.5 cm. high) illuminated from one end (*cf.* Clarke, 1932). After the usual phototropic reactions to light had been tested, the animal was removed from the trough and placed in a dilute solution of strychnine sulfate in pond water (1:200 of the saturated strychnine solution). After a few minutes the animal was transferred back to the trough and its responses again tested. This procedure was repeated until the animal became indifferent to the light stimulus.

During the repetitive tests *Daphnia magna*<sup>1</sup> which were originally negatively phototropic become constantly photopositive to all light intensities after 6 to 10 minutes in the strychnine solution. Animals which were originally primarily photopositive remain constantly positive. In both cases the usual responses to *changes* of illumination are abolished; neither an increase nor decrease of intensity of light produced any reaction. The orientation of the animal (its back toward the light) is not altered at any time. After a total stay of about 15 minutes in strychnine the animals begin to somersault, and soon fail to show any phototropic swimming movements. The occurrence of this condition probably accounts for the report that strychnine destroys phototropism in *Daphnia* (Moore, 1912). If the animals

<sup>1</sup> This and the other species tested were kindly furnished by Prof. A. M. Banta from his cultures.

are left longer in the strychnine, tetanic spasms follow and continue until the animals die after about 6 hours.

In the case of *Daphnia longispina* all animals tested were found to be primarily positively phototropic to all light intensities. Like *Daphnia magna* they became temporarily photonegative following sudden increase of light intensity. After 3 to 5 minutes in strychnine solution of the same concentration, the positive phototropism is destroyed. Although the animals tend to swim away from the light, the swimming movements are no longer normal, a good deal of somersaulting being exhibited. Rapid changes of light intensity now evoke no response. Longer sojourns in strychnine result in more frequent somersaulting and soon all phototropic movements are abolished.

### III

The galvanotropism of *Daphnia* was investigated by placing the animals in a trough of pond water similar to the one described above and passing a galvanic current through the water by means of zinc electrodes embedded in cotton pads at each end. The observations had to be made in a dark room, to avoid any conflict between a galvanotropic and a phototropic response. The trough was illuminated by a weak light placed just above the trough. When subjected to current densities of 0.5 to 1.0 milliamperes per cm.<sup>2</sup>, *Daphnia magna* exhibits a strong anodic galvanotropism. The reaction is very much more vigorous when the circuit is first closed. To a continuous current *Daphnia* becomes progressively less responsive until the current density is increased or the circuit is broken and made again. This is probably due to polarization within the animal. To weaker currents the organism is indifferent, whereas stronger currents produce general tetanic contraction which prevents the animal from swimming. Under these conditions the *Daphnia* sinks to the bottom, but it always lies on its back with the head toward the cathode. When the direction of the current is reversed, the animal turns itself around.

After 6 to 10 minutes in the strychnine solution *Daphnia* becomes strongly cathodic. The orientation to the direction of the current, however, remains the same. The body is tilted backward and the *Daphnia* progresses thus toward the cathode (Fig. 1). When subjected to stronger currents, the animal falls to the bottom lying on its

back with its head to the cathode exactly as before. This condition with reversed sign of galvanotropism but with orientation unchanged persists until tetanic spasms begin after about 30 minutes. Strychninized animals generally die after 1 hour when repeatedly exposed to the current. This is very much faster than in the case of the experiments involving stimulation by light.

In *Daphnia longispina* the galvanotropic responses were found to be essentially the same as in *Daphnia magna*. *Moina macrocopa*, however, exhibits a strong cathodic response. A current of 2.0 to 2.5 milliamperes per cm.<sup>2</sup> is required in this case. Treatment with strychnine does not alter the sign of galvanotropism and even after 30 minutes in strychnine solution *Moina* is still cathodic. This form, however, is so small that the position of the body during orientation cannot be observed with the naked eye.

#### IV

These experiments show that strychnine has a pronounced effect upon the sign of phototropism and of galvanotropism, but the orientation of the animal is not affected in either case. Thus strong support is provided for the assumption that the mechanism for *orientation* is distinct from the mechanism responsible for the *sign of phototropism*.

Confirmatory evidence was also obtained for the second assumption, that the antennae are controlled by reciprocally innervated antagonistic muscles. When strychnine is successfully employed as a means of testing for reciprocal innervation, the altered response of the neuromuscular system to a given stimulus is usually regarded as due to a "reversal of inhibition" in the reflex arcs (Sherrington, 1906). There are, however, other possible explanations for different cases: the drug might affect directly the receptors concerned (*cf.* Crozier and Federighi, 1924-25), or produce a reversal of tropism without the occurrence of simultaneous contraction of antagonistic muscles (Crozier, 1920); or, finally, strychnine might increase irritability to such an extent that stimuli normally producing a simple reflex, produce tetanic contractions of the whole muscular system similar to that in the "start" reflex (Cushny, 1919).

Although these other interpretations of the strychnine effect have not been excluded, the "reversal of inhibition" theory seems to offer

the most satisfactory explanation of the results with *Daphnia*. According to this view the effect of the drug is to change normal reflex inhibition into excitation, with the result that both sets of antagonistic muscles are stimulated to contract simultaneously. The stronger set of muscles is believed to overpower the weaker set. *Daphnia* treated with strychnine would respond to a stimulus by simultaneous contraction of both sets of muscles in the antennae. Since strychnine produces positive phototropism, the conclusion follows that the set of muscles producing the back or photopositive position of the antennae is stronger than the set producing the forward or photonegative position. A change in illumination no longer produces a change of the sign of phototropism because both sets of muscles are already in a state of increased tonus and the stronger continues to maintain the animal in the photopositive posture. Those specimens of *Daphnia* which are normally primarily photopositive continue to show the same phototropic response after strychninization. This condition can be understood on the same basis, for, if the back set of muscles is the stronger, they will control the sign of the reaction whether or not the forward set is also stimulated to contract.

The results of the experiments on galvanotropism may be interpreted in the same way. The orientation mechanism is not affected by strychnine and hence the *Daphnia* always faces in the same direction,—toward the anode. Normally the animal swims with the forward set of antennal muscles contracted and the backward set inhibited and relaxed. After a short stay in the strychnine solution, however, both sets of muscles are stimulated to contract, and, if the back muscles are the stronger, according to the deduction made above, the animal would be forced to swim tilted backward. The result is that *Daphnia* now moves to the cathode although it continues to face in the opposite direction.

#### SUMMARY

1. Experiments with strychnine were performed to test two assumptions important in the development of a theory for the mechanisms involved in the tropisms exhibited by *Daphnia*.

2. After a brief interval in strychnine solution *Daphnia* exhibits a reversal of the primary sign (*a*) of phototropism, from negative to

positive; and (b) of galvanotropism, from anodic to cathodic. In both cases the orientation of the body remains the same.

3. The mechanism responsible for the sign of phototropism and galvanotropism in *Daphnia* is therefore distinct from that underlying orientation.

4. Evidence is obtained indicating that changes in sign of tropism, produced by changes in illumination or by subsection to strychnine, involve the control of antagonistic muscles in the swimming appendages which are reciprocally innervated.

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# OSMOTIC RELATIONSHIPS IN THE HEN'S EGG, AS DETERMINED BY COLLIGATIVE PROPERTIES OF YOLK AND WHITE

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The osmotic relationships between the yolk and white of the hen's egg have recently aroused considerable interest because of the fundamental bearing of the observed results on the general problem of cellular energetics. Straub (25) first called attention to the fact that to maintain the observed freezing point difference between the yolk and white, the yolk must in some way produce an osmotic pressure difference of nearly 2 atmospheres, since, in spite of the difference in freezing points, the yolk membrane is freely permeable to water. This delicate membrane could obviously withstand no such mechanical pressure, so that it was assumed that the difference in pressure must be maintained by the constant application of metabolic energy at the membrane in some unknown manner. Straub assumed that the cell must derive the required energy from oxidative processes at the cell membrane. The problem of how such a steady state might be maintained would be of great interest in view of its bearing on water exchanges and secretion in living systems generally.

Hill (10) promptly confirmed Straub's observation of an osmotic pressure difference by means of thermal measurements of vapor pressures. However, Hill found that the observed difference was still present after the eggs had been kept for 29 days in hydrogen, and concluded that hence the source of the energy which maintained this non-equilibrium condition or steady state could not be oxidative in nature.

The question has also been studied in a series of papers by J. and D. Needham, Stephenson, Smith, and Shepherd (18-20, 23, 24). These authors find that without bacterial contamination the anaerobic lactate production of the hen's egg is so low that it would be necessary

to use all of the energy evolved to maintain the supposed osmotic difference. The isolated vitelline membrane showed no tendency to maintain osmotic non-equilibrium when separating saline solutions. From this and other lines of evidence the authors concluded that their results pointed against the idea that thermodynamic work was done by the membrane, and that no evidence could be obtained in favor of the alternative of an impermeability of the vitelline membrane to water.

The actual validity of the observation of an osmotic pressure difference was denied by Grollman (8) who showed that the true freezing point of the egg yolk was not easily obtained, and, finding that dialysates from yolk and white were in osmotic equilibrium, concluded that the observations of Straub and Hill were experimental artifacts and that no osmotic difference actually existed between yolk and white.

Meyerhof (17), on the other hand, on the basis of dialysis experiments, supported the view that an osmotic pressure difference existed; however, his experiments using the vitelline membrane do not agree with those using collodion membranes, but indicate a considerably smaller freezing point depression for the yolk. To explain this he invokes an idea previously used by Needham, that the outer yolk layer had a lower osmotic pressure than the inner. There is no adequate evidence that such a layer exists, and in any case it would be difficult to explain how such a situation might arise.

The disagreement on this important point indicated that a further study would be of interest. Not only are there two views as to the existence of the osmotic pressure difference between yolk and white, but among those who conclude that it exists, there is no agreement as to its significance or as to the method by which it is maintained.

Accordingly the osmotic relations between the yolk and white of the egg have been studied by means of freezing point and vapor pressure determinations, and by dialysis experiments. Certain previously unrecognized sources of error are described. It has been found that there is no osmotic pressure difference between the yolk and white of the hen's egg. This is in accordance with Grollman's observation and, furthermore, explains the failure of Needham and collaborators to detect the performance of any osmotic work by the yolk membrane.

The interest in the supposed action at the yolk membrane lay in the

fact that it appeared to be an easily accessible biological system with a single non-cellular membrane which was analogous to secretory epithelial tissues in being able to use metabolic energy to maintain or create an osmotic pressure difference. Although the egg yolk-white system does not possess this property, many cellular tissues do, and the mechanism of such tissue action remains a problem of fundamental interest.

*The Direct Determination of the Freezing Point of Egg Yolk*

Straub (25), and previously Bialascewicz (2) and others (22, 24) have reported the freezing point of egg white as  $-0.43$  to  $-0.46$ , that of the yolk  $-0.60$  to  $-0.56^{\circ}\text{C}$ . None of these authors give any detailed discussion of the technique used in their freezing point determinations, or of the range of experimental error involved. They also all appear to have overlooked a paper by Atkins (1) who gives the freezing point of the hen's egg as  $-0.454^{\circ}$ , and states that "no difference was found in the freezing point of the white and yolk of the same egg, and a mixture of white and yolk gave the same depression." Atkins describes his technique in detail, having worked with a minimum of supercooling and having approached the plateau of the freezing point from both higher and lower temperatures. Furthermore, Grollman, using an electrometric method, reports experiments in freezing the yolk in which he failed to obtain a clear-cut plateau in the temperature-time curve (8) such as should be obtained at the freezing point, and showed that very variable temperature maxima were obtained according to the degree of supercooling. He concluded that due to its high viscosity and low water content the true freezing point of the yolk could not be determined directly by the usual Beckmann technique or by the thermoelectric method which he used.

However, by the use of a technique recently described by Johlin (11) it has been possible to freeze egg yolk and to obtain reproducible plateaus of temperature which can be maintained for 10 to 30 minutes, and coincide with the values similarly obtained for egg white. The method consists essentially of efficient stirring and strictly limited supercooling of not over  $0.4^{\circ}\text{C}$ .

The method, as described by Johlin, was closely adhered to except that in order to make stirring possible about 10 cc. of yolk was used in a larger tube (17 mm.

inside diameter) instead of 1 cc. of solution, as used by him. The apparatus consists essentially of a small tube surrounded by a double air jacket in a cooling bath. Cooling mixture may be sucked up into the inner air jacket and then allowed to recede when the desired cooling has been attained. Seeding and local supercooling are brought about by adding small rings of nichrome wire cooled in solid  $\text{CO}_2$ . On exposure to air the rings become covered with a layer of frost, which acts as a small amount of finely dispersed centers for ice formation. The amount of water added is negligible, as determined by Johlin, but the local supercooling caused by the cold wire makes these rings a very efficient method of starting ice formation. Stirring was accomplished by moving the thermometer vigorously up and down a distance of a cm. or less. The stirring was more efficient than with the usual Beckmann technique, and this is an advantage for a mixture such as egg yolk which freezes with difficulty.

It has been stated that vigorous stirring may give too high a freezing point, but this could only be so if the temperature plateau was not determined as such. Stirring undoubtedly adds heat to the mixture, but in the presence of ice and solution, addition or loss of heat should change the volume of the ice phase, not the temperature. If an efficient mixing is maintained, a prolonged temperature plateau could only occur at an actual invariant point, unless the rate of heat addition exactly balanced the rate of heat diffusion. Stirring was done by hand and was somewhat irregular, so that it was impossible that the heat added could have balanced the heat lost for any appreciable time and hence a plateau determined over a fair range of time could not have been shifted significantly.

The thermometer zero point was determined in a mixture of ice and water. Determinations on NaCl and KCl solutions checked values given in International Critical Tables to within  $0.002^\circ\text{C}$ . The egg white freezes very readily, and no difficulty in obtaining a constant temperature was encountered when using fresh eggs. The yolk is inclined to give deceptive apparent freezing points at various inconstant temperatures below the freezing point of the white, which could account for the results reported by previous investigators. However, by avoiding too great supercooling, with vigorous stirring and repeated seeding constant temperature plateaus may readily be obtained at the freezing point of the white. These plateaus with the yolk check each other within a few thousandths of a degree, whereas the lower pseudo-plateaus are not reproducible. Pseudoplateaus do not always occur, sometimes the yolk goes promptly to its true freezing point.

Examples of actual determinations on yolk are shown in Fig. 1.

Eggs were obtained from Tancred hens (a white Leghorn breed), and were used within 20 to 40 hours after laying. Freezing points of the yolks and whites from six eggs are given in Table I. The values for the yolk represent the mean of three plateau values concordant to within a few thousandths of a degree. The plateaus were each fol-

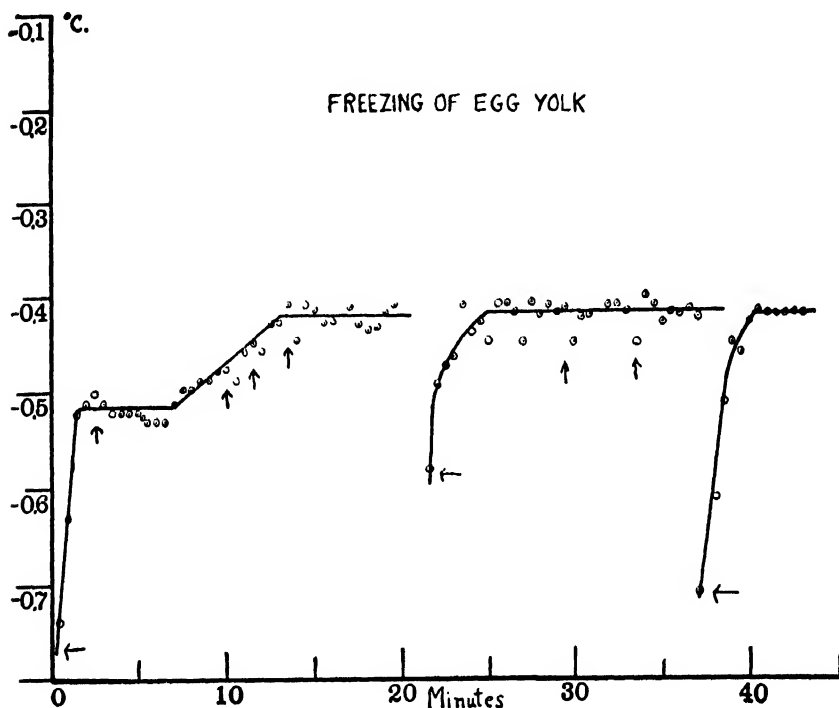


FIG. 1. Typical determination of the freezing point of a sample of egg yolk. Ordinates are the temperatures in degrees Centigrade, and abscissae are time in minutes. The first curve shows an initial rapid rise of temperature with the appearance of an apparent plateau which is followed by a gradual rise of temperature to a final plateau representing the freezing point. The second and third curves show repetitions of the process in which the final plateau appears without evidence of any secondary plateau. The arrows mark the points where the yolk was seeded.

lowed for 3 to 10 minutes and the value for the plateau is the average of readings taken every 30 seconds. These individual readings had a probable variation within 1 to 2 hundredths of a degree, dependant somewhat on the rate of stirring. The readings for the white were

taken usually from one or two plateaus, as no difficulty in obtaining the true freezing point was encountered with the white. The observed average values for the yolk and white differ by only  $0.001^{\circ}\text{C.}$ , as given in Table I.

It is concluded that there is no significant difference in the freezing point depression of the yolk and white of the hen's egg. The yolk is difficult to freeze not only because of its high viscosity, low water content (48 per cent), and high content of fat globules which have low heat conductance, but also probably because it contains a dialyzable material which decreases the rate of ice crystal formation, as will be described in the next section.

TABLE I  
*Freezing Points of Yolk and White of Hens' Eggs*

Egg No.	Yolk	White	Difference
	$^{\circ}\text{C.}$	$^{\circ}\text{C.}$	$^{\circ}\text{C.}$
1	-0.422	-0.434	-0.012
2	0.452	0.438	+0.014
3	0.424	0.426	-0.002
4	0.416	0.414	+0.002
5	0.419	0.419	0.000
6	0.418	0.412	+0.006
Average.....	0.425	0.424	

The value of  $-0.425^{\circ}\text{C.}$  found in the freezing point of egg white is in agreement with the results of Rice and Young, but is somewhat higher than that reported by previous investigators (Straub,  $-0.46$ , Atkins,  $0.454$ , Grollman,  $0.456$ , Smith and Shepherd,  $0.452$ ). This might be accounted for by either of the following reasons. The value  $0.425$  was obtained from eggs which were used within 20 to 40 hours after laying. Eggs of this lot which had been kept at  $1^{\circ}\text{C.}$  for 6 weeks still tasted perfectly fresh but had a freezing point of  $-0.46$  for yolk and white, an increase which is probably due chiefly to evaporation of water. Table eggs bought at another store and apparently perfectly fresh had a freezing point of  $0.447$ . Mature yolks removed from the ovaries of two hens had depressions of  $0.408$  and  $0.420$  respectively, so that the lower values are to be preferred as representing the conditions

in freshly laid eggs. The use of a larger freezing tube similar to that used in the Beckmann apparatus gave somewhat greater values ( $0.03^{\circ}$ ) for the freezing point depression of white than were obtained in Johlin's apparatus, a difference probably due to the more efficient stirring obtained in the latter. The freezing of the white was performed in a tube of 10 mm. diameter containing a thermometer of 9 mm. diameter, so that very efficient stirring was produced by moving the thermometer. We might thus attribute the slightly higher freezing points of egg white observed by previous authors to the use of older eggs or to an inefficient stirring during the determination. Eggs of different breeds according to Rice and Young vary slightly as regards their freezing points.

The present findings of the same freezing point for the yolk and the white are in agreement with the early observation of Atkins (1), and it is concluded that the observations of Straub, and others (2, 22, 24, 25), were in error due to their failure to recognize that the determination of the true freezing point of egg yolk is beset with technical difficulties.

#### *The Osmotic Pressure of Egg Yolk as Determined by Dialysis*

In order to confirm the direct determination of the freezing point of egg yolk just described, observations were made on the freezing points of solutions which were allowed to come into equilibrium with the yolk across collodion membranes. Freezing point determinations were made by the same method used for egg yolk, except that volumes of only 2 cc. were used, in a tube of appropriate dimensions. The greater fluidity of the dialysate permits a much more efficient stirring than is possible with egg yolk.

It was found that it was often more difficult to obtain the actual freezing point of the dialysate than of the yolk itself. In following the temperature-time curve, after seeding the supercooled solution, an initial rapid rise of temperature in the first  $\frac{1}{2}$  minute is followed by a marked change in the freezing rate and a very slow attainment of the final equilibrium temperature.

Typical results obtained in freezing dialysates are shown in Figs. 2 and 3. In Fig. 2, Curve B represents the freezing of a dialysate in which, after the initial rapid rise of temperature during the first half minute after seeding, there succeeded a slight but steady rise of temper-



ature for 10 minutes or more, to a moderately well defined plateau terminated by an abrupt temperature rise. Curve C shows the same type of behavior except that an apparent plateau appears during the first 2 minutes, after which the temperature gradually rises to the final plateau. The freezing of a 1.00 per cent NaCl solution is shown in Curve A, where it is seen that a plateau is reached after about 3 minutes, which is more stable than that of the dialysates, and is terminated

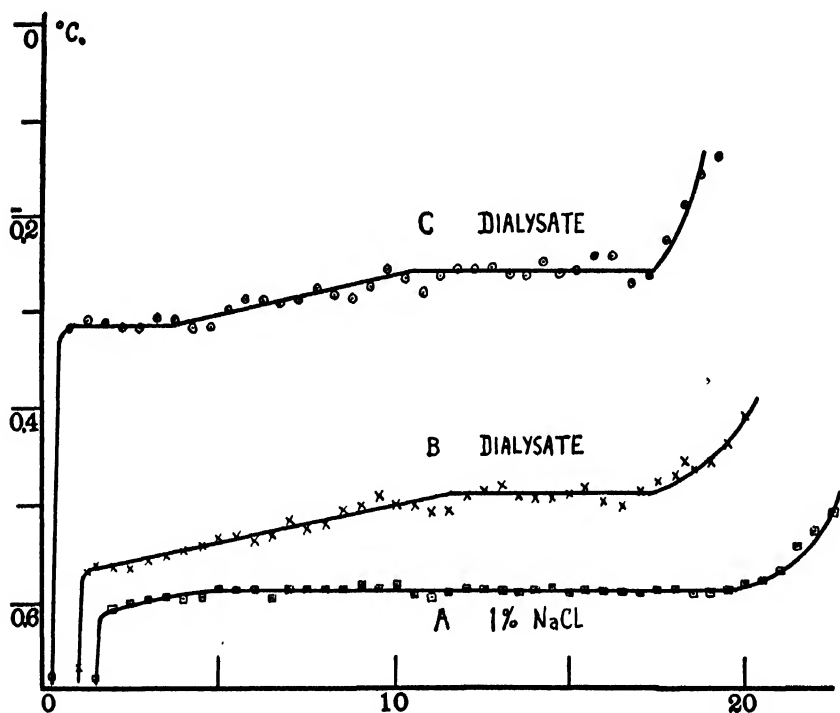


FIG. 2. The freezing of egg yolk dialysates compared with a 1 per cent NaCl solution. Ordinates are temperatures in degrees Centigrade: abscissae, time in minutes. The three curves have the same origin, but are displaced laterally for convenience in representation. It will be seen that, following the rise in temperature after seeding the supercooled solutions, a constant temperature is attained more rapidly by the NaCl solution than by the dialysates.

in the same way by an abrupt temperature rise. In the case of the NaCl solution the heat liberated by freezing results in a prompt rise to a temperature at which equilibrium is maintained between the ice and the solution. Heat is continuously added by the friction of stirring, which gradually melts the ice, and when the ice phase is completely

melted a further rise in temperature ensues. In the case of the dialysates it is evident that the attainment of thermal equilibrium between the ice and the original solution is delayed. It is obvious from these curves that the true freezing points of the dialysates are less easy to determine with accuracy than is that of the NaCl solution.

Fig. 3 shows an even more anomalous type of freezing, which was frequently encountered with dialysates. It is seen that the change in

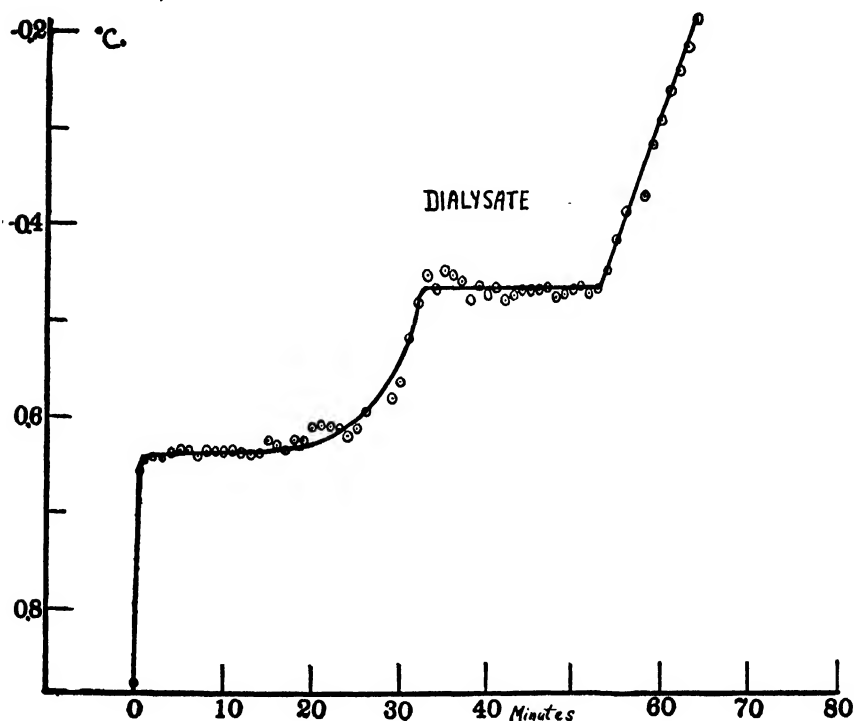


FIG. 3. The freezing of an egg yolk dialysate in which a secondary low plateau appears before the true freezing point is reached. (Ordinates, temperature in degrees Centigrade, abscissae, time in minutes after the initiation of the freezing process.) In freezing dialysates there were obtained various types of temperature-time curves similar to or intermediate between those shown in this figure and in Fig. 2.

freezing rate which occurs after the first  $\frac{1}{2}$  minute is followed by a plateau which is later succeeded quite abruptly by a second plateau at a higher temperature. Various curves intermediate between those of Figs. 2 and 3 are obtained, so that often a distinct initial plateau is

succeeded gradually by the final plateau which is terminated by the disappearance of the ice.

The lower plateau varies, in dialysates of different concentrations, according to the temperature of the final plateau, so that it cannot represent a eutectic or other invariant point. Such a secondary inflection has never been observed in freezing NaCl or KCl solutions.

The lower temperature plateau cannot represent the true freezing point because it is followed by a higher plateau before the ice phase has disappeared. The lower plateau is associated with the presence of large amounts of ice, which would concentrate the solution, and hence lower the temperature at which the liquid phase is in equilibrium with ice. The form of the curve is such that one might be misled and consider the initial plateau as representing the true freezing point if one did not follow the temperature until the final disappearance of ice. If one does follow the temperature until the ice disappears, one finds that the plateau which is presented just before the ice goes is never attained promptly, but only after a gradual rise of temperature during which at times a lower plateau appears. As long as ice is present in a solution, and stirring is adequate to maintain thermal equilibrium, the temperature of the solution cannot exceed its true freezing point.

It is only when a small amount of ice is present that the concentration of the solution is not affected, and it is only under these conditions that the true freezing point is obtainable. This state of affairs occurs in the region of the second plateau just before the abrupt rise following the melting of the last ice crystals. Hence one must take the true freezing point of a solution determined by this method to be the highest temperature which is maintained at a constant level in the presence of ice.

The freezing behavior of the dialysate suggests that a substance is present which slows the rate of ice formation. This has been shown to be the case in the following manner. Direct measurements of the rate of formation of ice crystals have been carried out according to a procedure due to Gernez (5), and previously applied to aqueous solutions by Walton, Brann, and Judd (26, 27, 3). Two U-tubes containing saline and dialysate respectively were immersed in a cooling bath, and after seeding at one end, the times were obtained at which the ice crystal boundary had moved a distance of 40 cm. In making a

determination, the tubes of saline and dialysate were always seeded simultaneously. The velocities are a function of temperature, and were obtained at several temperatures. The results are given in Table II, the velocities being expressed in cm. per second. It will be seen that the crystallization velocity in the yolk dialysate is less than in an NaCl solution of the same freezing point, with an average difference of 25 per cent. Whether this decrease in crystallization velocity is the only factor responsible for the anomalous freezing of the dialysates cannot be decided at present. It is, however, interesting to note that  $MgCl_2$  solutions, which according to Brann slow the crystallization velocity of water, show an inflection in the temperature-time curve when freezing which is quite similar to the inflection of the dialysate curves.

A change of crystallization velocity after the first  $\frac{1}{2}$  minute has been described by Marc (12-16) in the crystallization of a series of salts in water solutions. The first  $\frac{1}{2}$  minute of freezing was characterized by a greater velocity constant than the remainder of the process. Marc attributes this to changes at the crystal solution interphase which acquires certain constant properties after the first  $\frac{1}{2}$  minute. Marc (14-16) adduces evidence that crystallization velocities are affected by means of the adsorption of inhibiting substances at the crystal surface. Brann, on the other hand, related the effect to solvation of the solute in a series of salts; whether the mechanism is essentially the same in the two cases is not clear at present.

In the light of the above experiments, it is probable that anomalous freezing behavior, such as shown by the egg yolk dialysate, is a phenomenon of fairly general occurrence. The true explanation of this anomaly must probably await a more extensive knowledge of the mechanism of crystallization from solutions.

In the present determinations, it was found that repeated freezing of the same dialysate gave values for the final plateau (determined by averaging the individual readings) which checked to within  $0.01^\circ C$ . The approach to the plateau varied in the same dialysate between the two extreme types of curves shown in Figs. 2 and 3. The point of initial inflection of the curve was constant to within about  $0.02^\circ C$ . so that this anomaly is a fairly constant characteristic of a given solution. What substance in the yolk is responsible for this effect has not been

determined. It is present in dialysates which are clear and colorless. The anomalous freezing is not affected by boiling the dialysate. At-

TABLE II

*Crystallization Velocities in Cm. Per Sec. of Egg Yolk Dialysate and Isosmotic NaCl Solution*

Bath temperature	0.71 per cent NaCl	Dialysate
°C.		
6 mm. tube		
-2.12	0.228	0.173
1.3	0.049	0.041
1.9	0.192	0.155
10 mm. tube		
2.3	0.286	0.270
1.8	0.153	0.105
2.0	0.198	0.106
1.9	0.176	0.109
Average.....	0.182	0.137

TABLE III

*The Freezing Point of Egg Yolk Calculated from That of Its Dialysate*

A. Against 1 per cent NaCl: membranes soaked in 0.71 per cent NaCl				
Weight yolk	Membrane weight	Volume dialysate	Δ Dialysate observed	Δ Yolk calculated
gm.	gm.	cc.	°C.	°C.
19.6	0.9	10	-0.503	-0.420
24.2	0.35	4	0.467	0.426
23.8	0.3	4	0.456	0.410
26.2	0.3	7	0.491	0.437
27.7	0.5	7	0.487	0.434
B. Against H <sub>2</sub> O: membranes soaked in H <sub>2</sub> O				
25.1	0.4	5	0.304	0.440
27.4	0.52	5	0.298	0.423
Average.....				0.427

tempts to adsorb the material on carbon and cotton seemed to accelerate the freezing somewhat, but were not effective enough to remove the inflection in the freezing curve. The interfering substance was present

in dialysates after using the following types of membranes: cellophane, a synthetic parchment, gold-beaters' skin, and collodion membranes made by drying to various degrees with or without ethylene glycol. The interfering substance is not evident in the white of fresh eggs, but in the white of eggs which had stood for 6 weeks the anomalous freezing may be very marked.

When the freezing temperature was determined by following the temperature-time curve with constant stirring until the abrupt rise of final ice disappearance occurs, the dialysate freezing points shown in Table III were obtained. For the experiments in Table III collodion membranes were made following, in principle, the method of Pierce (21).

Merck's C.P. collodion to which was added 1 per cent ethylene glycol was poured into test-tubes ( $15 \times 1.8$  cm.) which were rotated slightly and dried overnight. Membranes were then washed for 50 hours in running water, and placed in 0.71 per cent NaCl (where indicated) overnight. The membrane with included solution was weighed just before adding egg yolk, and the weight  $\times 0.8$  (membrane water content as determined by drying) taken as the weight of contained solution in computing the freezing point of the yolk from the value of the dialysate. The water content of the yolk was found to average 48 per cent. Dialysis against 1 per cent NaCl was allowed to continue for from 15 to 20 hours, with gentle shaking, at room temperature. No difference in the results was obtained after 6 hours dialysis. In dialyzing against water, experiments were run for 50 hours at  $1^{\circ}\text{C}$ . No significant differences in the osmotic pressure of the yolk at the two temperatures were observed.

The average value of the freezing point of the yolk calculated from that of the dialysate agrees with that obtained by direct measurement with a difference of only  $0.002^{\circ}\text{C}$ .

This dialysis evidence that yolk and white are in osmotic equilibrium is in agreement with Grollman's dialysis experiments and in opposition to Straub's and Meyerhof's conclusions from dialysis. It is worthy of emphasis that the true freezing points of egg yolk dialysates are not always obtained without difficulty, and unless this source of error is recognized one may obtain erratic results.

#### *Vapor Pressure Measurements*

In order to further verify the conclusions based upon freezing point data a series of vapor pressure determinations on egg yolk were per-

formed. In this way freezing points of egg yolk could be calculated from data based on an entirely different type of experimental technique. For this purpose the dynamic method of Washburn and Heuse (28) was utilized, as used by Grollman (6) for the determination of the vapor pressure of dog's blood. I am indebted to Dr. Grollman for performing these determinations of vapor pressure.

The method as used in these experiments consists essentially of passing a stream of gas over the material to be investigated with which it comes into equilibrium. The gas saturated with the vapor from the unknown solution is then led over an absorber which removes the water vapor. The dried gas is next led through a similar train in series with the first where it becomes saturated with water vapor from a known solution, and it then gives up this water vapor to a second absorber. By weighing the absorbers before and after the experiment a differential measure of the vapor pressure of the unknown solution is obtained.

The saturators were of the type described by Washburn and Heuse (28). They consisted of a train of glass tubes placed on a rocking table, and were about half filled with liquid. In the absorbers the gas was passed through a condensation bulb, an  $\text{H}_2\text{SO}_4$  train, and Dehydrite. The gas (air or oxygen) was led first over a presaturator containing 1 per cent NaCl, before being introduced into saturators containing solutions to be tested, to avoid concentration by drying of the solutions. The equilibration temperature was  $20^\circ$ .

Several technical difficulties were encountered in carrying out determinations on egg yolk which rendered the results more erratic and open to greater error than that encountered by Grollman in his study of blood. The viscosity of egg yolk prevents an efficient shaking of the material and hence tends to prevent true equilibrium occurring between the gas and the yolk. To avoid this occurrence a slower passage of the gas was required. The passage of the gas was continued for about 20 hours, but was not extended further because of the danger of putrefaction. The tendency of egg white to foam rendered necessary the insertion of a trap at the end of the saturator containing this substance, to minimize any carrying over of small droplets of solution into the absorbers. Both of these sources of error would lead to obtaining too high values for the freezing point depression of egg yolk.

The freezing points were calculated from the vapor pressure data by means of the simplified equation of Callendar (4) which is sufficiently accurate when the freezing point depressions are less than  $1.0^\circ\text{C}$ .

$$\log e \frac{p_o}{p} = \frac{LF_o \Delta T_F}{RT_o^2} = 0.0097 \Delta T_F$$

where  $\Delta T_F$  is the difference in the freezing points of the two solutions,  $LF_o$  is the molar heat of fusion of the pure solvent at its freezing point,  $T_o$  the absolute freez-

ing point of the known solution, and  $p_0$  and  $p$  the vapor pressures of the known and unknown solutions respectively.

In this calculation of freezing point data from vapor pressure differences determined at temperatures other than the freezing point, it is assumed that we can neglect the change in the heat of dilution of the solution with temperature and the change in the activity with temperature. As discussed by Grollman (6) these factors are negligible for NaCl up to 1 M concentration, and we have no knowledge that they are otherwise for the constituents of egg yolk.

The results are given in Table IV. There is considerable experimental error as indicated in the range of the values, but the average calculated freezing point of  $-0.45^\circ\text{C}$ . shows quite satisfactory agree-

TABLE IV  
*The Freezing Point of Egg Yolk Calculated from Its Vapor Pressure*

Experiment No.	Solution against which yolk was measured	Freezing point of solution assumed	Difference between freezing point of yolk and Column 2	Calculated freezing point of yolk
		$^\circ\text{C}$ .	$^\circ\text{C}$ .	$^\circ\text{C}$ .
1	Egg white	-0.43	-0.05	-0.38
2	" "	0.43	+0.06	0.49
3	" "	0.43	+0.03	0.46
4	0.75 per cent NaCl	0.44	+0.05	0.49
5	" " " "	0.44	-0.04	0.40
6	H <sub>2</sub> O	0.00	+0.47	0.47
Average.....				0.45

ment with the value obtained directly. It is slightly high, but the chief sources of error discussed above would tend to give too great a freezing point depression.

These results are in opposition to those obtained by Hill (9) using a vapor pressure method (10) which depends on the measurement of the differences in the heat of condensation of vapor in two solutions. The method appears to be admirable for dilute aqueous solutions, as shown by Grollman (7), but the disparity between Hill's determinations on the egg and the various lines of evidence presented herewith leads me to believe that Hill's method cannot be applicable to a concentrated viscous mixture such as egg yolk, especially since the present determinations show that the expected osmotic equilibria between egg yolk



and white are fulfilled, whereas Hill's results require an explanation *de novo*.<sup>1</sup>

#### SUMMARY

The osmotic pressure of the yolk and white of the hen's egg have been shown to be identical, by means of direct freezing point determinations, dialyses, and vapor pressure measurements.

Dialysates of egg yolk slow the rate of ice formation compared with NaCl solutions. They also show a marked change of freezing rate as the freezing point is approached. The anomalous freezing behavior of this material may lead to errors in the determination of the true freezing point which would tend to make the value for the yolk erroneously low.

The postulate of a vital activity at the yolk membrane maintaining an osmotic pressure difference is thus shown to be unnecessary, since a simple osmotic equilibrium exists between the yolk and the white.

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<sup>1</sup>While this paper was in press an article appeared (Bateman, J. B., 1932, *J. Exp. Biol.*, **9**, 322) purporting to confirm Hill's vapor pressure determinations on the egg. In view of the present experiments it is impossible to accept Bateman's measurements as representing the true vapor pressure difference between yolk and white, and consequently I suspect that some unknown factor produces a pseudoequilibrium of heat quantities in the thermopile experiments—probably conditions at the yolk-air interphase are not identical with those in the body of the material. Significantly, Bateman's findings of the vapor pressure of mixtures of yolk and white are in disagreement with his own determinations of the yolk vapor pressure, but agree with the value of approximately  $-0.42^{\circ}\text{C}$ . freezing point depression of both yolk and white.

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# AUTOMATIC RECORDING OF MOVEMENTS OF PLANT ORGANS

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In the study of many reactions of plants involving slow displacements of organs in space, one is often confronted with the necessity of keeping track of the successive positions occupied at definite moments by the organ considered as well as making a record of them free of personal bias. Photographic recording seems to be the most adequate procedure although it may present a certain number of difficulties. The chief difficulty has been, until lately, the necessity of operating for most cases under very dim red light, inactinic to the plants but unfortunately also to most photographic plates. Developments of the technique of supersensitization of panchromatic plates, following the work of Adams and Haller (1920), and Dundon, Schoen, and Briggs (1926), on the cyanins, has very considerably reduced this obstacle; and the possibility of finding on the market plates highly sensitive to the red end of the spectrum and in general rather uniform as regards speed and homogeneity provides an invaluable aid.

That such records, free from instrumental or personal aberrations, are needed even for very much investigated questions, is shown abundantly by the conflicting statements that one finds, for instance, in the literature of such subjects as geotropic curvature of plants. Different authors working on what often seems to be the same material, interpret their observations in quite different ways.

The study of the geotropic reaction in coleoptiles of *Avena* led us to develop the method here described. One could perhaps use the so called "time lapse" photographic process (Buder, 1926) using one of the highly perfected motion picture cameras. But we had it in mind to get a photograph of about natural size and often even enlarged directly on the record. In many cases also where measurement is

important it may be very difficult to ascertain, in the time lapse photographs, the identity of position of the film in relation to sprockets or driving mechanism on successive exposures.

Finally, as the development of this work made apparent to us, the camera must be versatile in its possible ways of recording. For instance, we may need at some moment to record a phenomenon involving an upward curvature and at another moment a right-to-left bending.

All these reasons led us to set up the following device, which has proved highly successful in its operation. It is based essentially on the possibility of photographing under very dim red light to which the plant is insensitive, on a plate specially sensitized to the red end of the spectrum.<sup>1</sup>

If the light is turned on and off automatically at regular intervals of time, we may keep the shutter of the objective constantly open; of course the whole apparatus must be completely shielded from outside light.

The apparatus consists of the following parts (Fig. 1).

*Camera.*—A long extension camera of about 40 to 45 cm. working distance between focal plane of objective and ground glass, is provided with an objective (Zeiss Unar of  $f/5.n.a.$  and 155 mm. focal distance) which under these conditions is highly luminous and has enough depth of focus to take care of any possible motion of the plant organ (here, the coleoptile of oats) in planes different from the one perpendicular to the axis of the optical system. In most cases the iris diaphragm reduces the  $n.a.$  to  $f/9.0$ . Two types of plate holders are provided: the first is a standard type, taking a plate  $83 \times 108$  mm. ( $3\frac{1}{2} \times 4\frac{1}{2}$  inches); the second is built to take a plate  $102 \times 254$  mm. ( $4 \times 10$  inches) in a brass frame which can slide easily between brass rails on a reinforced aluminum plate, the center of which is provided with a square opening, of the same size as the frame of the ground glass end of the camera. This special plate holder is built to fit the square end of the camera in a vertical or horizontal position, to permit taking records with a plate moving vertically or horizontally at constant speed. The displacement of the plate is obtained by means of a device described a little later.

With both types of plate holders the recording is done by turning on a source of dim, red light, inactinic for the plants, for short constant durations, at constant (but adjustable) intervals of time, the shutter being always kept open. It is

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<sup>1</sup> We used extensively the Wratten and Wainwright panchromatic plates sensitized with neocyanine and later the same maker's hypersensitive panchromatic plates.

necessary to shield the recorder completely from all light for this reason. When the light source is turned off, the whole device is in darkness.

*Light Sources.*—We used glass cylinders, 65 mm. in diameter containing a saturated aqueous solution of fuchsin as a screen for a long 40 watt bulb of the showcase type (with a single filament). A light-tight cap holds the socket of the lamp in place and seals the mouth of the glass cylinder, reducing evaporation of the solvent. The outside of the cylinders is lacquered heavily with a black lacquer

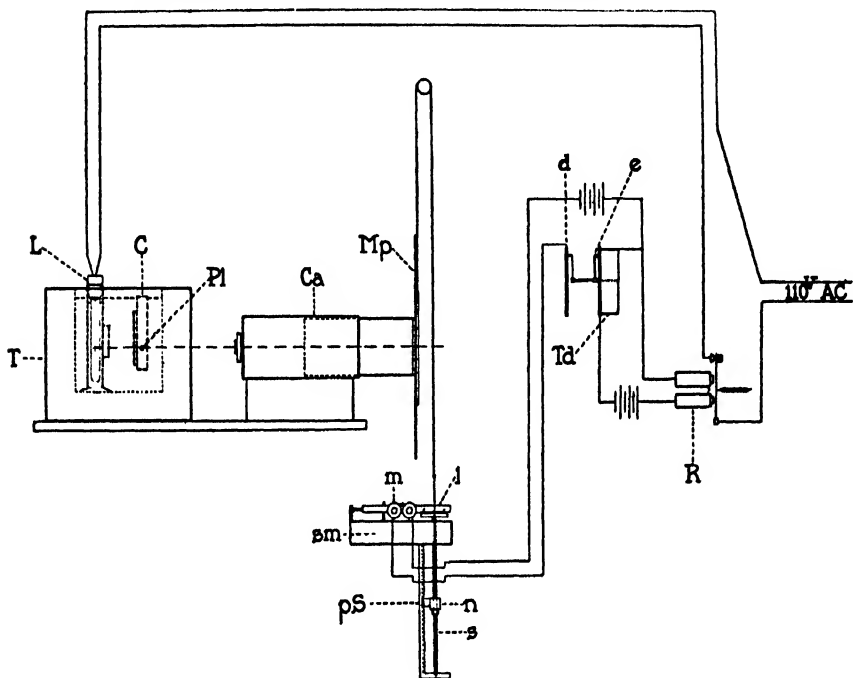


FIG. 1. The recording apparatus. *T*, thermostat with lamp *L* and cell *C* containing the plant *Pl*. *Ca*, photographic camera with moving plate holder *Mp*. *pS*, plate holder shifter with magnet *m* and curved lever *l*, conditioning the travel of the nut *n* along the micrometric screw *s*, put in movement by the spring motor *sm*. *Td*, timing device with contacts for exposure *e* and contacts for displacement of the plate *d*. *R*, relay.

to make it light-proof. One window  $10 \times 40$  mm. is cut in the black cover of the cylinder. It is covered by a small frame holding a ground glass to diffuse the light. Two sources of light of this description are placed symmetrically in the thermostat tank, at about 9 to 10 cm. behind and to each side of the experimental plant. The plant is contained in a special cell whose back is made with a Corning G-24 plate (deep red filter), transmitting only light of wave lengths above  $620 \text{ m}\mu$ . Care is taken to avoid direct penetration of the beams of light into the camera. As the sensitivity of *Avena* according to Blaauw seems to be almost nil

at 530 m $\mu$ , and as no phototropic bending has been seen even after 2 to 4 hours' exposure, this lighting system is entirely satisfactory.

*Relay System.*—The lights are turned on and off through the medium of a clock whose minute hand is arranged to brush over copper strips sunk in a disk of hard rubber at intervals of 30° apart. Every passage of the brush over the strip of metal closes the circuit on a relay (operating on 2 volts) which is able to carry the load of the lighting circuit on its contact points. This relay prevents any pitting of the clock contacts, which would vary the duration of illumination. The same clock by means of an insulated extension of the minute shaft actuates also the automatic shifter of the long plate holder; the contacts for this circuit are entirely separated from the illuminating circuit and are adjustable so as to fall between times of exposure of the plate.

*Plate Holder Shifter.*—The carrier consists essentially in a long micrometric screw, one end of which is provided with a circular plate on which up to 20 pegs can be inserted; these are caught by a curved lever when no electrical current flows through the coils of an electromagnet. When the current flows through this magnet, the attracted lever liberates one peg and the circular plate turns until the next peg is stopped. This movement of the circular plate is determined by a powerful spring, strong enough to enable a nut to travel upward along the micrometric screw, being led by a tongue-and-groove arrangement in the stand supporting the screw. To a stirrup solidly made of fine steel rods fixed to the nut is attached a flexible, braided wire which passes over a pulley fixed to the ceiling. To the other end of the wire is attached the moving plate holder. Any amount of travel imparted to the nut allows the plate holder to move by the same amount.

As any number of pegs can be placed in the plate, and as a large number of electrical contacts can be inserted in the second circuit of the clock, the distance over which the plate can be made to move between each exposure may be varied from a fraction of a millimeter to about 11 mm.

*Cell for Plant.*—The seedling,<sup>2</sup> grown in sterile sawdust in a small glass tube 25  $\times$  15 mm., is placed for experimentation in a container, the front of which is made of plate glass; the back is of a piece of Corning G 24 glass and the sides are of brass pieces soldered together to form a frame. The pieces of glass are cemented to the brass by Picc $\ddot{e}$ n.

The total capacity of this cell is about 480 cc.; this is slightly decreased by the presence of the movable brass mounting stand for the glass tube. The stand is so made as to permit rotation of the seedling around a horizontal axis by means of a 90° gear. With addition of a set of reducing gears this device can be used as a clinostat. Usually it is employed for orienting the seedling while the seedling is still vertical. Two brass pins soldered to the sides of the cell assure the correct position of the mounting for every experiment. A glass plate closes the cell after

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<sup>2</sup> In most of the cases no marks are made on the seedling; sometimes we placed small India ink dots at known distances from the tip.

replacement of the mounting stand. It has been found very useful to coat the inner side of the front plate glass with one of the products sold for preventing condensation of water on glass surfaces, especially in the cases where one keeps the atmosphere of the cell saturated or nearly saturated with water vapor.

### RESULTS

With the apparatus described it is possible to obtain two types of photographic record. The first, "the standing plate picture" is especially adapted for determination of time course relationships. For instance, in the case of a coleoptile of *Avena* it enables one to obtain clean-cut records of the time course of geotropic curvature. In



FIG. 2. A "standing plate" picture of the coleoptile of *Avena* bending geotropically; successive exposures every 30 minutes;  $1.75 \times$  natural size.

records of this type all successive images are superimposed one on top of another; all immobile parts will therefore be represented by one trace, in opposition to any moving part which will be reproduced as separate images placed one above or below another. Up to twenty-five successive exposures can be made on the same plate without blurring out the different individual exposures. Such an image describes automatically the course of events as related to time (*cf.* Fig. 2). It records successive positions of the motile portion of an organ in respect to a fixed portion, no motion being imparted to the plate; we then know the relation existing between these positions and time in a synthetic way. It dispenses with the necessary orientation of



each exposure in relation to a common base line, an operation which is likely to introduce large errors. The second type, "the moving plate picture," permits an analysis of each successive position and the respective shape of the organ (Fig. 3). By means of a comparator it is then easy to detect very small changes in shape or position if one

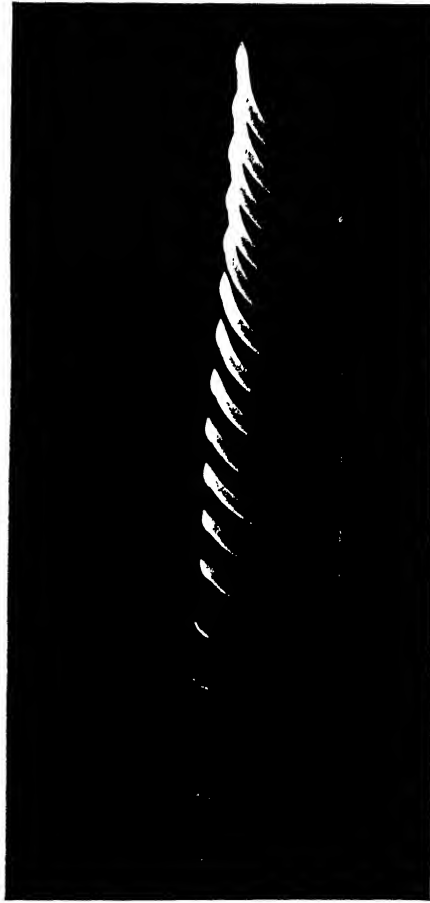


FIG. 3. A "moving plate" picture of a coleoptile of *Avena*. Exposures every 20 minutes.  $1.7 \times$  natural size.

has taken care to fix a pointer in the field. It makes possible, furthermore, the construction of composite pictures analogous to the one obtained by the standing plate method. It is especially well adapted to determinations of reaction times for geotropic excitation, as well as for recording growth of vertically oriented coleoptiles.

The two types of record supplement one another. Each has its special advantages. Both can be enlarged easily up to 9 or 10 diameters, which makes the total magnification up to twenty times. Measurements can be made in this way with an increased precision. The use of this method of recording will be demonstrated in an account of geotropic curvature of coleoptiles of *Avena*.

#### SUMMARY

An automatic photographic recording apparatus is described. It uses plates sensitized to the red end of the spectrum for recording movements of organs of plants which cannot be photographed in white light. It enables one to obtain two types of pictures:

1. A "standing plate" picture where the successive positions of the organ to be registered are superimposed on the same plate, no motion being imparted to the plate.
2. A "moving plate" picture where, by means of a clock-controlled plate shifter, each successive picture is entirely separate from the preceding one.

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# GEOTROPIC CURVATURE OF AVENA COLEOPTILES

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## I

Examination of the literature concerned with geotropic reactions in plants shows quite divergent statements about the rates of geotropic curvature exhibited by comparable organs in similar plants (*cf.*, for instance, Jost, 1923, II, p. 258). Coleoptiles of *Avena* have been used extensively for such studies. Maillefer (1910) describes the rate of curvature as gradually increasing with time, the increase being proportional to the square of the time, while Tröndle (1913) finds the rate of curvature to be constant. It is true that Tröndle's way of computing a rate of curvature smoothes out possibly existing divergences and that the procedure is highly objectionable.

On the other hand Lundegårdh (1917) finds it possible to divide the whole geotropic curvature into three phases: a "*Start Phase*" before the curving is actually visible with naked eye; a "*eumotorische Phase*" where the process goes on at constant rate, terminated abruptly by the onset of the "*Gegenreaktion*," the last phase.

From our own observations it is rather difficult to admit, in general, such a clean-cut subdivision of the geotropic reaction; furthermore, we are in disagreement with Lundegårdh as to the constancy of the rate of curvature, as we will later demonstrate. Most of the differences encountered probably originate in the difficulty of obtaining a record of the process free from personal or instrumental error. The apparatus developed by us for obtaining records of the process of geotropic curvature seems to be free from such defects, and it has proved serviceable in the present work. It was used to determine the steps involved in geotropic curvature in *Avena* as related to time, as well as to get more information as to the changes in the shape of the region near the tip of the coleoptile. This last point is of great interest in the determination of the "reaction time."

*Technique.*—For a description of the photographic recording apparatus we refer to our previous paper (1932). It will be sufficient here to say that the principle of the method used is to record automatically, on a plate sensitized to the red end of the spectrum, successive positions of the experimental plant at regularly spaced intervals; the plate may be either fixed (in which case only the moving part of the organism is recorded by images separated on the plate) or it may be moved at regular intervals of time (the records are totally separated in this case and are placed one above the other on the plate).

As we have encountered a few difficulties in obtaining straight coleoptiles of *Avena*, and as we found in some cases departures of our standard technique to be influencing to a certain extent the exact reproducibility of the results, we may describe our procedure in some detail.

The seeds used were of a pure strain of *Avena*, the so called "Cornellian oats."<sup>1</sup> An examination of the dry seeds showed them to be remarkably uniform in external characters; we took care, nevertheless, to eliminate the infrequent seeds which were smaller than the normal in any one lot taken at random. They were kept in an air-tight glass container which prevented water loss. These seeds, after removal of the seed coat, were planted in sterile maple sawdust (*cf.* Navez, 1929) contained in glass vials 25 mm. high by 15 mm. in diameter, the sawdust retaining 4.3 times its weight of water. The seed itself is planted dry, with the embryo side upwards, the axis of the seed being inclined about 10° from the vertical and the tip of the seed being about 2 mm. above the sawdust level (the rim of the vessel). Germination proceeds in a light-tight, ventilated germination box, flat-black inside, in an air thermostat kept at 22° or 22.5°C. The vials are fixed in a wooden base provided with holes where the vials fit loosely.

After 72 hours the seedlings are ready for use; the coleoptiles are then 25 mm. long on the average; they are at the period of maximum growth rate; *viz.*, on the almost straight portion of their growth curve. The departures from this length are small, of the order of  $\pm 2$  mm. At this moment the seedling, in its vial, is fixed in a vertical position on an adjustable mounting stand. The adjustable stand is then returned to its normal position (determined by two brass pins) in the cell kept in a water thermostat and the moment of rotation of the seedling through an arc of 90° considered as time 0. All these manipulations are done under the very dim red light provided by a Wratten safe-light lamp with a Series 2 filter and a 20 watt bulb. The lower spectral limit of such light is 635 m $\mu$  and is thus of a wave length considered as inducing no phototropic effects in the seedling. The first photographic record is taken as soon as possible; *i.e.*, within 30 seconds of the time of turning the seedling horizontally. In the experiments here reported the temperature of the thermostat where the plants were reacting was adjusted to be

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<sup>1</sup> We are very much indebted to Dr. W. T. Craig of the Agricultural Experiment Station, Cornell University, Ithaca, N. Y., for supplying us with this strain.

equal to that of the germination thermostat (22° or 22.5°C.); this reduced the time of thermal adaptation to a minimum. Furthermore the dark room where the thermostats are kept was maintained at about the same temperature. The atmosphere of the glass cell where the geotropic reaction took place, and of the germination box, was adjusted at 75 to 80 per cent of the saturation point in water vapor. With this technique it is possible to obtain without special difficulty coleoptiles of *Avena* of uniform length and behavior; the results obtained with such material show only very small variations.

## II

Examination of Fig. 2 of the preceding paper shows immediately that we may distinguish two periods in the geotropic response: the pure geotropic reaction, and the autotropic oscillations. In none of our records have we detected any mixture of these two effects occurring simultaneously, so long as we work with our pure strain of *Avena*. Our strain shows the two reactions clearly separated in time. This is not true of all strains; in a few trials with some seeds of unknown origin we have found the two manifestations conflicting one with another.

In the subsequent treatment of our data, we will consider only the true geotropic curvature.

*Shape of the Curved Coleoptile.*—All reacting seedlings showed a smooth, rounded curving which in the successive stages shortens its radius of curvature. In no case have we observed an almost straight tip separated from a straight base by a very short curved portion. The fact that such "curvatures" have been observed and represented by reliable workers makes it highly probable that some specific structural differences in diverse races can affect the external appearance of the bent coleoptile.

Examination of one of the pictures (Fig. 1) that we may consider as typical,—or, what is better, working on a photographic enlargement of such a picture—shows that the upper and lower profiles of the coleoptile are not one and the same curve merely shifted; this is especially so in the region of the tip. In other words, if we wish to deal with curvatures of such stems, we have to define a reference line. For theoretical convenience we have chosen for reference the axis line, the *neutral axis* of the coleoptile, with the meaning used in work on the

resistance of materials.<sup>2</sup> In the pictures this neutral axis is easily determined through the symmetrical construction of the coleoptile: the neutral axis must be equidistant at each point from both upper and lower profiles. In further discussion this line is the one used, unless specifically stated otherwise. As far as can be determined, for all the pictures of bending coleoptiles obtained, the successive neutral axes of the bending part of the stem correspond very nearly to arcs of circles rather than to portions of hyperbolas or parabolas (Fig. 1). Such arcs of circles can be defined by the positions of their centers, the radii, and the angle subtended by the arc.

The locus of the centers of curvature during the progress of bending is an arc of a curve of parabolic type; it is not a straight line perpendicular to the neutral axis at the point where the curved portion of the neutral axis becomes tangent to the horizontal straight part of the same axis, as might be expected for the simple case. The departure from simple expectation can be attributed to two causes, which may add their effects, namely (1) the stem is growing while the bending takes place; (2) the stem shows a certain amount of viscous elasticity or shearing; one might think of the first of these two causes as the main factor affecting the locus of the centers of curvature, since elongation does occur.

The radius of curvature varies also with time; Fig. 2 shows the type of relation obtained. But as in the first stages of curvature the radii are very large and very difficult to measure with accuracy, the first observation points of Fig. 2 are subject to a probable error which must be large. The angle subtended by the curving part is readily determined by measuring the angles that the tangents to the neutral axis at

<sup>2</sup> The neutral axis is a more logical reference line than one of the profiles (as used by Dolk, 1930, p. 63), for two reasons: (1) the two profiles are not the same curve merely shifted; (2) the diameter of the coleoptile varies with the distance from the tip. The same objections apply also to the method used by Dolk for determining the curvature of different regions of the stems. The graphs given in his memoir (*cf.* his Figs. 8 to 25) correspond to radii of the concave profile and are not necessarily identical in time relation with those which can be obtained for the other side, especially when using Dolk's method of measuring the radii. We have found such difficulties in the use of this method when we applied it to our seedlings as to preclude accurate measure of the curvatures of the stems.

the tip of the stem make with the horizontal. By a simple geometrical construction one can readily see that in the case of arcs of circles the angles at the center and those just defined are equal. This procedure has the advantage of relieving us from determining the centers of curvature—or the locus of these centers—and substitutes for it the

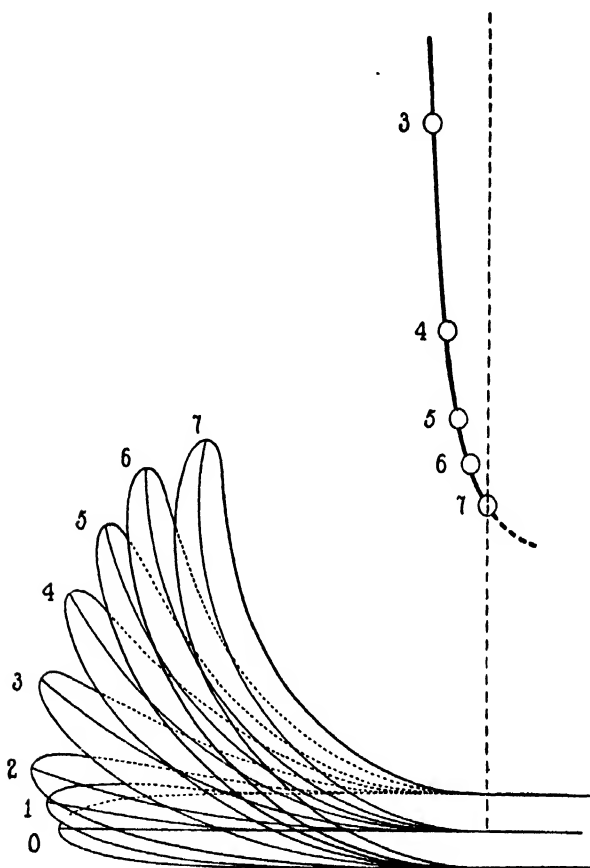


FIG. 1. Successive positions of the neutral axis of a geotropically curving coleoptile at 30 minute intervals of time, and locus of the centers of curvature of these arcs.

measurement of a tangent to an arc. As can be seen in Fig. 3, the change of this angle with time is not haphazard: it is best described by a smooth sigmoid curve having a point of inflection at about 42 per cent of total completion. This curve gives us also a way of defining a "rate of bending" which may be less arbitrary than the "rates of



curvature" as used in the literature.<sup>3</sup> Angular displacement of the tip and linear displacement of the tip have been used to give such "rate" curves. It is obvious that even on a photograph, especially if reproduced by direct contact without enlargement, it is very difficult to measure with any precision the angle that the tip makes with the horizontal. This is particularly so when the coleoptile bends in a

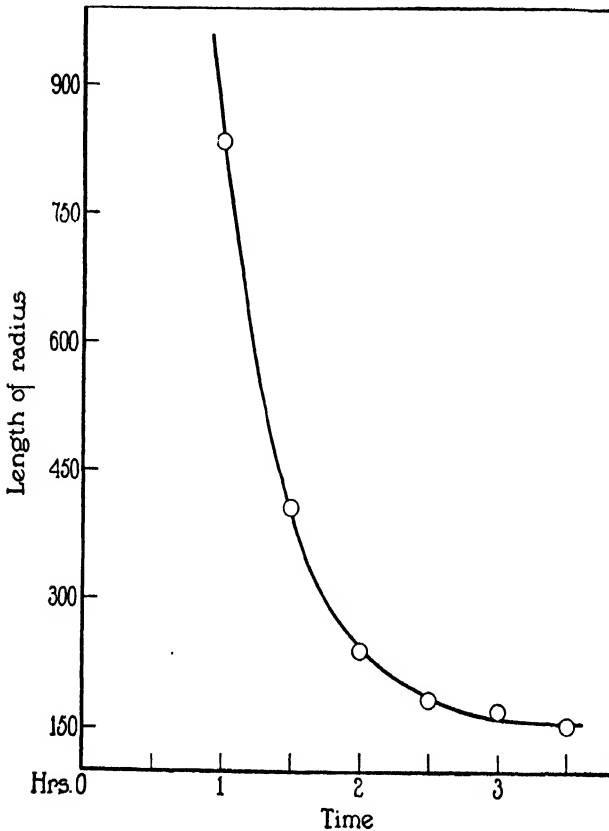


FIG. 2. The relation of radii of curvature to time

smooth, rounded curve without any straight portion near the tip. As to the estimation of this angle by visual observation, we think that the experimental error is so great as to make this measure almost

<sup>3</sup> This curve gives us a relative measure of the amount of curving tissue, as we know for any position of the neutral axis the corresponding radius of curvature and the angle subtended by the curved part.

useless. The linear displacement of the tip (as used by Brauner, 1922, for instance), measured from the point occupied by this tip at zero time as origin, is also inadequate, because of the lack of correspondence between an arc and its chord for angles of the magnitude here observed.

The derivative of the Curve A in Fig. 3 gives us a more reasonable way of defining a "rate of curvature." It is apparent from Curves A and B that the curvature is not proceeding at a constant rate, as it has been described, and that it is difficult to divide the curve into 3 parts as Lundegårdh has done. We can only state that the rate of

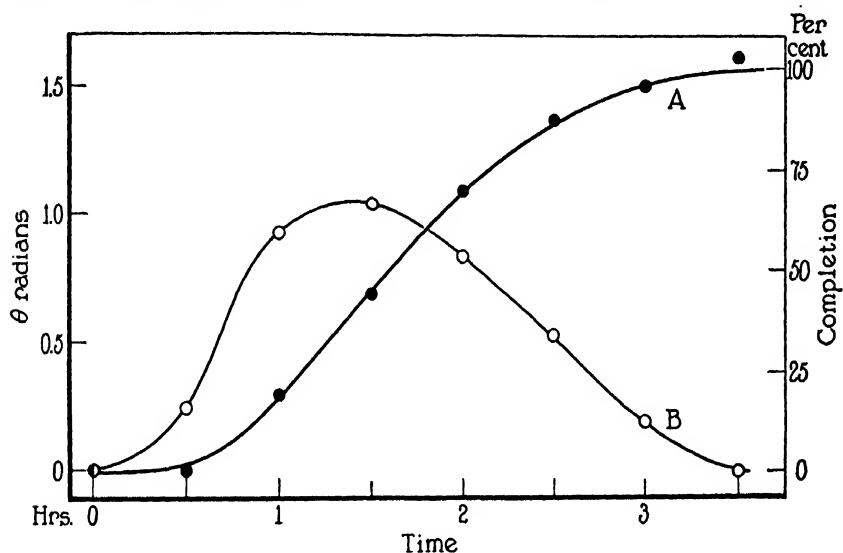


FIG. 3. Curve A, the time relation of the angle made by the tangent to the neutral axis at the tip with the horizontal.

Curve B, the derivative of Curve A, showing the variation in the "rate of curvature."

curvature at first increases with time, passes through a fairly well defined maximum at about 90 minutes after the beginning of the experiment, under these conditions, and then gradually decreases to zero.

Fig. 4 shows that for different seedlings grown under the same conditions the derivative curves are in good agreement. Similarity between curves for single seedlings shows obviously that the lack of sharply defined periods in the reaction is not a matter of smoothing out by averaging different curves, but is intrinsic.

*First Steps in the Geotropic Response.*—In a few pictures we observed that before any upward bending occurs, a slight drop of the tip of the coleoptile takes place. The fact that the base of the coleoptile remains exactly in its original position excludes the idea of a shift of the organ due to packing or displacement of the sawdust in which the seed grows.

As this phenomenon was not of constant occurrence we must conclude that it is probably not a fundamental step in the geotropic reaction, but that more likely we have to do with a nutational movement. Perhaps it is a passive sagging of the tip of the coleoptile, progressing

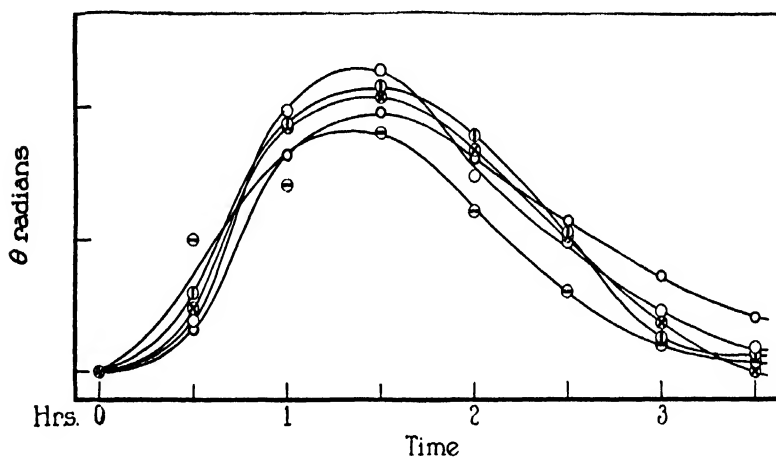


FIG. 4. A set of derivative curves (as in Fig. 3, B) showing the similarity of the course of the reaction with individual seedlings.

until the true geotropic response occurs. Bose (1906) and Maillefer (1910) are probably the only authors who have investigated this bending, but, despite the extensive tables published by Maillefer, we cannot be convinced that the sagging is the first step in the geotropic response; the phenomenon occurs in only  $\frac{2}{3}$  of his observations. Even then the amplitude of the downward bending is often of the order of magnitude of the nutational movements described in the literature. In any case we would consider it as a mere mechanical sagging rather than a short phase of positive geotropic response rapidly reversed by the true geotropic bending.

## III

*Geogrowth Reaction*

Opinions as to a "geogrowth reaction" are divided: one group of observers admits, the other rejects the idea that on geotropic stimulation the normal rate of elongation of the coleoptile is affected.

As pointed out before, the period of utilization of the coleoptiles, in our work, corresponds to the almost straight part of their growth curve.

The total duration for the geotropic reaction here studied is about 3 hours. If such a thing as geogrowth reaction exists, it will obviously be shown best by the way in which the neutral axis of the coleoptile changes with time. This change, as well as that of the upper and lower profiles, can be very easily measured on our plates. The fixity of both plate and object in respect to one another removes the difficulty of ascertaining the fixity of a reference point from which to measure elongation. This point is taken at 22 to 25 mm. from the tip, in a region where virtually no growth is found during the period of geotropic reaction. The length of each line considered is measured on the enlargement by means of a precision map measurer; the precision attained in successive readings of the same length is of the order of 1 part in 400.

As shown in Fig. 5 there is no apparent "growth reaction," either positive or negative. The Curve A, relating elongation of the neutral axis to time, is a straight line over the 3 to 4 hour period during which the geotropic reaction occurs. The fact that before and after the moment the plant is stimulated elongation proceeds at the same rate also precludes the existence of a lag period for this hypothetical geogrowth reaction, analogous to the one occurring on light stimulation (Blaauw, 1914).

Another point which also clearly comes out of the analysis of our pictures is the relative rate of elongation of the upper and lower profiles. As depicted by Curve B in Fig. 5, the upper side slows down its rate of elongation by an amount nearly equal to the one by which the lower side (Curve C) increases its rate. But in no case have we found complete inhibition of growth on the upper profile. At most we find a partial inhibition. These results depart from a good many

reported, perhaps for the reason that in our experiments no other significant stimulus was acting at the same time as the geotropic vector. All direct contacts with the plant being avoided, the method is free from interference by accidental stimulation. Very likely we are justified in attributing to such technical defects some of the results reported by previous authors.

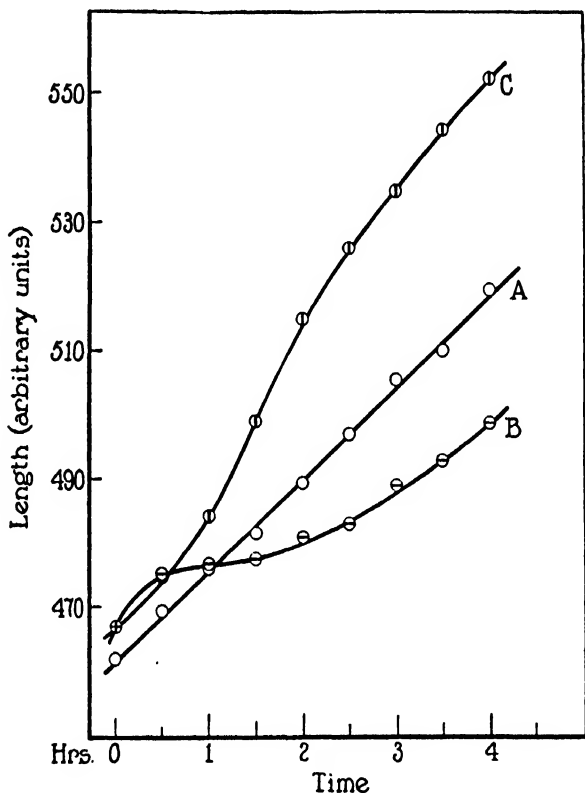


FIG. 5. Curve A, elongation of the neutral axis during geotropic response.  
 Curve B, elongation of the upper profile of the same coleoptile during geotropic reaction.  
 Curve C, elongation of the lower profile during reaction.

#### IV

#### *Curvature and Distribution of Growth Substance*

How do the preceding observations fit in with the hormonal theory of the geotropic curvature? In experiments performed by one of us (A. E. N.) we have tried to correlate the angle made by the tip with the

distribution of growth substance between upper and lower half of the tip. The technique used was essentially that of Went (1928) and Dolk (1930), using a 3 per cent agar-gel for extraction of growth substance; the lengths of the tips cut from the reacting plants were between 7 and 10 mm. long; the duration of contact for diffusion into the agar was always 60 minutes.

Care was taken to rub the little razor blade used for prevention of cross diffusion between both halves, before use, with a small piece of paraffin which left on it a very thin coat of this substance sufficient to suppress capillary creeping of the tissue juice. The blade protruded about 1 mm. above the small blocks of agar used to receive the diffusate. (For full details concerning this technique the reader is referred to the papers of Went and Dolk.) On the assumption that the curvature is determined by unequal growth on the upper and lower flanks of the horizontally placed stem we ought to find a definite correlation between the differences in amounts of substance in the upper and lower halves of the coleoptile, and the angle through which the stem has bent. In other words, it is when the tip of the coleoptile is placed horizontally that the difference should be maximum;<sup>4</sup> as the tip is gradually brought back to verticality the difference should decrease and reach zero when the tip is again vertical. The ratio of the amounts of growth substance present in the two halves ought therefore also to vary accordingly with the angle made by the tip with the vertical.

In the set of experiments here briefly reported, we did not have a large enough number of observations to enable us to treat the data statistically; they will be amplified in a subsequent paper and are given here only as a corollary to the discussion of angular displacement with time.

In Table I the amount of substance present in symmetrical halves of tips is expressed in terms of degrees, indicating the amount of deflection observed in prepared standard coleoptiles of *Avena* when supplied with a standard block of agar containing the diffusate from the tip of the coleoptile under measurement. We know from the experiments of previous investigators, and our own, that for a tip placed vertically

<sup>4</sup> Of course, this largest difference is not instantaneous. It can reach its maximum only after a lapse of time sufficient for shifting the growth substance from its state of equidistribution has been allowed. A "lag period" is thus expected.

we have an equal distribution of growth substance in what we would call "upper" and "lower" halves if the coleoptile were placed horizontally. With four tips placed on the same agar plate, this amount

TABLE I

The growth substance diffusing from tips of vertical coleoptiles is equally distributed between the two halves of the tip.

No. of tips	"Upper"	"Lower"
4	13.0°	14.0°
4	17.0°	19.0°
4	16.0°	13.0°

TABLE II

The amount of growth substance diffusing from upper and lower halves of tips of coleoptiles of *Avena* placed horizontally and subsequently reacting, varies with time. The amounts are expressed in degrees of deflection of test coleoptiles (see test) after 90 minutes.

Angle of tip to the vertical	Duration of stimulation	No. of tips tested	Average deflection of test coleoptiles	
			"Upper" half	"Lower" half
90°	<i>min.</i>			
	15	6	7°	16°
	20	4	9°	16°
	20	8	4°	13°
60°	30	4	5°	18°
	75	4	11°	15°
		4	12°	16°
		6	10°	15°
30°	110	4	12°	14°
		6	8°	13°
		4	11°	15°
		3	12°	14°
0°	160	4	6°	7°
		4	12°	12°
		4	15°	16°

of substance, in three successive experiments, is represented by the figures in Table I.

For the experiments using tips of coleoptiles removed while the

stems are bending, the number of plants used for each experiment was variable and is given in the column "No. of tips tested" in Table II.

From Table II we can conclude that the distribution of growth substance is in itself directly influenced by gravity and that its distribution continues to be affected by this factor while the bending takes place. One may conceive that the apparent ratio of distribution is in part dependent on the utilization of the growth-promoting substance for further growth, and that its decrease ought to follow the progress in bending. The data thus far collected show in a qualitative way that such an assumption is valid.

#### SUMMARY

Applying a photographic recording method, and working on enlargements of the plates so obtained, the shape of the geotropically curving coleoptile of *Avena* was studied. This shape is expressed in terms of curvature of the neutral axis of the coleoptile; for the true geotropic response of the strain of *Avena* used, the curvature of the neutral axis is an arc of a circle. The "rate of curvature" is taken as the derivative of the curve relating time with the angle with the horizontal made by the tangent to the neutral axis at the tip. This rate increases up to a maximum and then decreases gradually. No "geogrowth" of the whole coleoptile is found. It is shown that the curvature is due to an increase in the elongation of the lower side of the horizontally placed coleoptile with a concomitant decrease of the rate of elongation of the upper side. This is correlated with a shift in distribution of "growth substance" in the tip as affected by change of position of the coleoptile.

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# PROTOPLASMIC POTENTIALS IN HALICYSTIS

## II. THE EFFECTS OF POTASSIUM ON TWO SPECIES WITH DIFFERENT SAPS

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In studying the potential differences in plant cells it is desirable to alter the solutions in contact with the protoplasm, not only at the outer surface, but at the inner or vacuolar surface as well, since this may differ from the outer in its properties. Such interior alteration may be done experimentally (to be described in other papers of this series), or by the comparison of different species essentially alike except for different vacuolar saps. A striking case of such a natural experiment is available in the two known American species of *Halicystis*. *H. Osterhoutii* of Bermuda has a cell sap differing only slightly from sea water in composition;<sup>1</sup> *H. ovalis*, of the Pacific Coast, on the contrary accumulates potassium (as chloride) to some thirty times its concentration in the sea water.<sup>2</sup> These two species differ otherwise only in minor details of morphology.<sup>3,4</sup> They thus offer an excellent opportunity to test the internal effect of a salt so generally active bioelectrically as potassium chloride.

Has *H. ovalis* a relatively high positive potential, like *Nitella*, as a result of the high potassium content of its sap? Or does it, like another marine plant, *Valonia*, have a small negative potential? What part of the observed potential is due to the potassium concentration gradient, what to an independent, internal gradient or asymmetry

<sup>1</sup> Blinks, L. R., and Jacques, A. G., *J. Gen. Physiol.*, 1929-30, **13**, 733.

<sup>2</sup> Brooks, S. C., *Proc. Soc. Exp. Biol. and Med.*, 1929-30, **27**, 209.

<sup>3</sup> Blinks, L. R., and Blinks, A. H., *Bull. Torrey Bot. Club*, 1930-31, **57**, 389.

<sup>4</sup> Smith, G. M., Observations on some siphonaceous green algae of the Monterey Peninsula, in Contributions to marine biology, Stanford University Press, 1930, 222.

in the protoplasm? Finally, of critical importance to the theory of accumulation of electrolytes, does potassium ion have a higher mobility in the protoplasm of *H. ovalis* where potassium is accumulated, than in *H. Osterhoutii* where it is not accumulated?

In answer to these questions, comparisons are given in this paper between the potentials observed with *H. Osterhoutii* and *H. ovalis* under similar experimental conditions.

### Method

The method of impalement and measurement has been described elsewhere in more detail.<sup>5</sup> Fine glass capillaries, filled with artificial sap, were inserted from

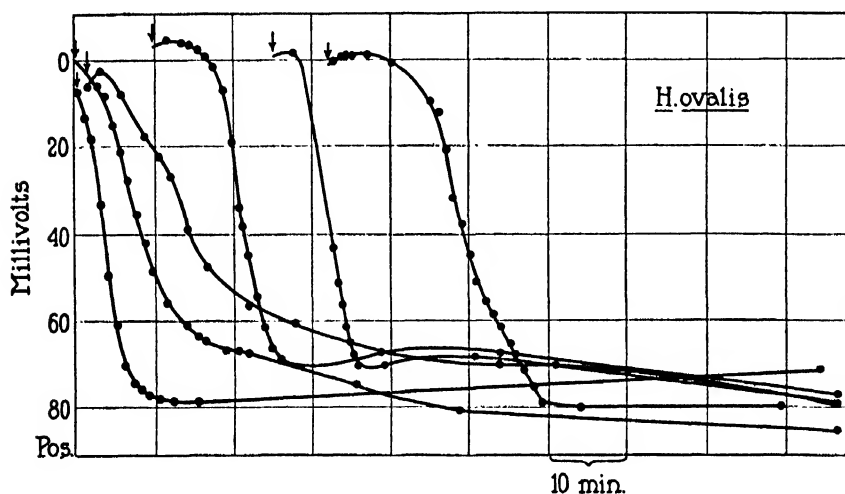


FIG. 1. Potential difference across the protoplasm of *Halicystis ovalis* immediately after impalement. The growth of P.D. in *H. Osterhoutii* is essentially the same. Cells in sea water.

above into the cells, which were supported below on glass rings.<sup>6</sup> Measurement was by compensation, the null instrument being a vacuum tube electrometer drawing no appreciable current. Saturated KCl-calomel electrodes with KCl salt bridges were employed for accurate measurements. For convenience in rapidly measuring a series of impaled cells, silver wire electrodes were sometimes used. These were frequently recoated electrolytically with chloride. They gave potentials not over 1 mv. apart when both were in equilibrium with sea water, or 2

<sup>5</sup> Blinks, L. R., *J. Gen. Physiol.*, 1929-30, **13**, 223.

<sup>6</sup> Damon, E. B., *J. Gen. Physiol.*, 1931-32, **15**, 525, gives an illustration of this arrangement for supporting cells.

or 3 mv. apart when one was in artificial sap. These corrections were applied to the readings. When natural sap was applied externally to the cells, it was found that the silver electrode could not be immersed directly therein without giving very erratic readings; the chloride was possibly reduced by some organic substance of the sap. (This would constitute a very serious objection to the insertion of silver electrodes, such as Sen's microelectrode,<sup>7</sup> directly into cells.) A guard tube was therefore employed in these cases, the electrode being kept in artificial sap, which communicated with the natural sap through an agar-filled capillary.

The cells were usually between 5 and 10 mm. in diameter, and frequently had a bit of the *Lithothamnium* substrate still attached to the pedicel. Cells in active reproduction were not used, but the potentials of those impaled a day or two after reproduction did not differ markedly from those which had recovered for longer periods. Subsequent reproduction did not usually occur while the cells were impaled, although they might live as long as 2 or 3 weeks, *i.e.*, over one reproductive period.

Since neither calcium nor magnesium content was included in the analyses<sup>2,8</sup> of *H. ovalis* sap available at the time of experimentation, artificial sap could only be made up approximately. This was done by mixing 1.5 parts of 0.5 M KCl with 1 part of sea water, giving the ratio  $K \div Na = 1.5$ , found by Brooks.<sup>2</sup> That this was a fair approximation was shown by the similar behavior of such artificial sap and of natural sap extracted from the cells.

Throughout this paper the potentials given are those of the outside solution, this being positive to the sap in the external circuit (positive current tending to flow outward across the protoplasm). In the figures these positive potentials are plotted below the zero line in conformity with the convention for *Valonia* and *Nitella* in this Laboratory. This is the reverse of the plotting used in the first paper<sup>5</sup> of this series. Change of solution is indicated by arrows.

### *Comparison of Potentials in Sea Water*

Immediately after the impalement of cells of either species of *Halicystis*, there is zero potential difference, or at most a few millivolts, measurable between sap and sea water. But (as shown for *H. ovalis* in Fig. 1), this rapidly increases, reaching in the course of 30 to 60 minutes a maximum which is then maintained during the life of the cell,—as long as 2 or 3 weeks. There are subsequent small fluctuations from day to day, but the potential of healthy cells seldom goes below 50 mv. (except when conditions are experimentally altered), nor does it much exceed 90 mv. In general the potential of *H. Osterhoutii* lies between 60 and 75 mv.; that of *H. ovalis* somewhat higher.

<sup>7</sup> Sen, B., *Proc. Soc. Exp. Biol. and Med.*, 1929–30, **27**, 310.

<sup>8</sup> Hollenberg, G. J., *J. Gen. Physiol.*, 1931–32, **15**, 751.

between 70 and 85 mv. The figures given in Table I were obtained from a group of several hundred readings for each species (all after at least 1 day's impalement); partly from routine determinations of all cells impaled at any given time (taken each day or two), partly from the initial readings obtained with cells before experimenta-

TABLE I

*Comparison of Potential Differences across the Protoplasm of Impaled Cells of Two Species of Halicystis*

	Mean value	Average deviation of readings	No. of readings	Probable error of mean
	mv.	mv.		mv.
<i>H. ovalis</i> (1931).....	79.7	$\pm 5.0$	270	$\pm 0.25$
<i>H. Osterhoutii</i> (1930).....	65.6	$\pm 9.1$	150	$\pm 0.63$
(1931-32).....	68.4	$\pm 4.2$	130	$\pm 0.3$

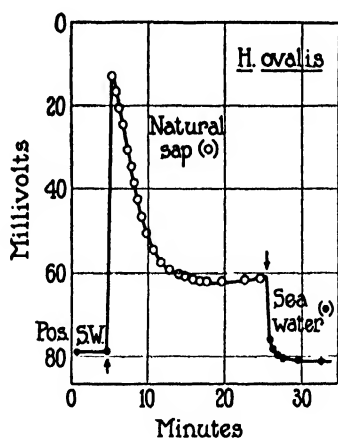


FIG. 2

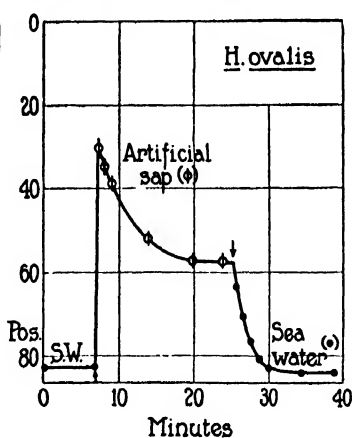


FIG. 3

FIG. 2. Potential difference across the protoplasm of *H. ovalis* in sea water and in freshly extracted natural sap.

FIG. 3. Potential difference across the protoplasm of *H. ovalis* in sea water and in artificial sap.

tion. The readings below 50 mv. were discarded in computing the mean, since almost invariably these were accompanied with evident injury, the cell usually dying the same day, with rapid decline of potential to zero. They thus represent, in the author's opinion, as nearly as possible a fair sample of healthy cells of each species. The

values for *H. Osterhoutii* for 1931–32 should be given more weight than those for 1930, since the technique was better, and the average deviation less. We assume therefore that the mean potential difference across the protoplasm of *H. ovalis* is about 12 mv. more positive than across that of *H. Osterhoutii*, each being immersed in the sea water of its accustomed environment and measured under the same general conditions of experimentation, *e.g.* temperature (15–20°C.), illumination (diffuse north light in the laboratory), pH (8.1), etc.

### *The External Application of Potassium Chloride*

Since the higher positive potential occurs in *H. ovalis*, the species containing the higher concentration of KCl, it is interesting to know

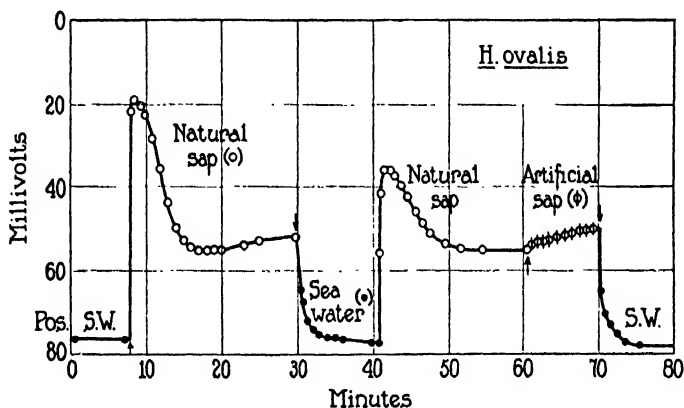


FIG. 4. Potential difference across the protoplasm of *H. ovalis* in sea water and during successive applications of natural sap. Artificial sap is substituted for natural sap during the second exposure.

how much of it is due to that salt. This is determined by abolishing the gradient of KCl concentration across the protoplasm. Solutions containing 0.3 M KCl (approximately the amount in the sap of *H. ovalis*) were applied (maintaining the necessary physiological balance with salts of calcium and magnesium). The effects of applying natural sap and artificial sap to *H. ovalis* are shown in Figs. 2 to 5. There is first a rapid decrease of potential, followed by a slower increase during which the potential is again built up, usually to about 60 mv., but in a few cases nearly equaling the original potential in sea water. Since this occurs when the potassium concentration is equal on both

sides of the protoplasm, the observed potential in sea water must be largely protoplasmic, the potassium potential contributing at equilibrium only 20 mv. out of nearly 80, and often less. Very similar

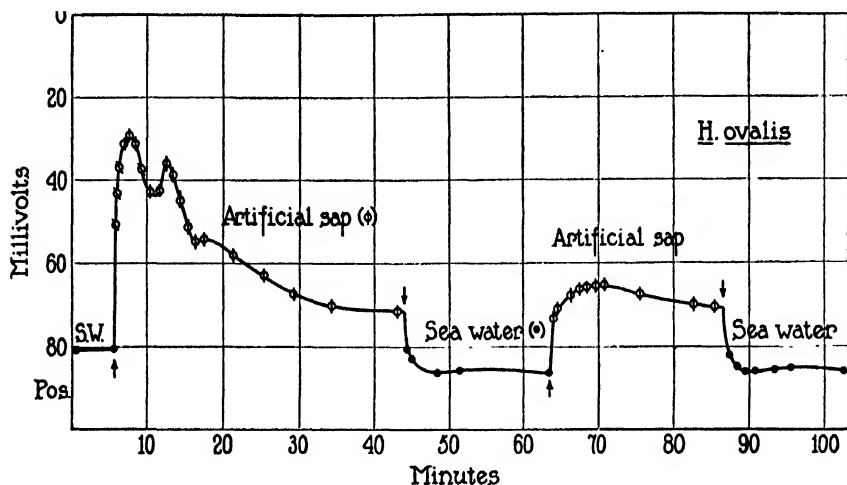


FIG. 5. Potential difference across the protoplasm of *H. ovalis* in sea water and artificial sap, showing reduced effect of second exposure.

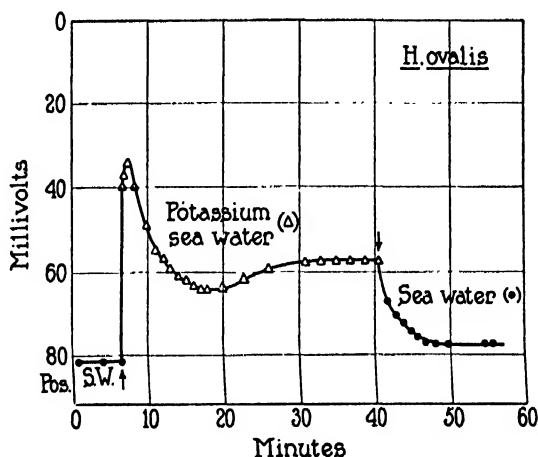


FIG. 6. Potential difference across the protoplasm of *H. ovalis* in normal sea water and in potassium sea water (artificial sea water in which potassium is substituted for sodium).

curves (Fig. 6) are obtained when potassium sea water (artificial sea water in which the sodium is entirely replaced by potassium so that  $KCl = 0.5 \text{ M}$ ) is applied externally, a large positive potential per-

sisting even though the gradient of potassium concentration is now reversed.

However, because the gradient of potassium chloride contributes only partially to the observed steady potential across the proto-

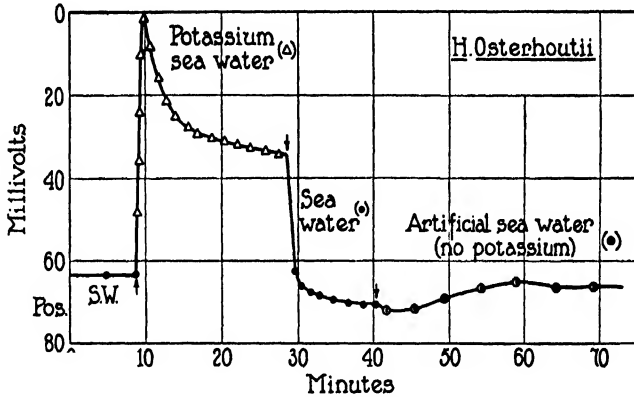


FIG. 7. Potential difference across the protoplasm of *H. Osterhoutii* in normal sea water and in artificial sea water containing (a) no sodium, (b) no potassium.

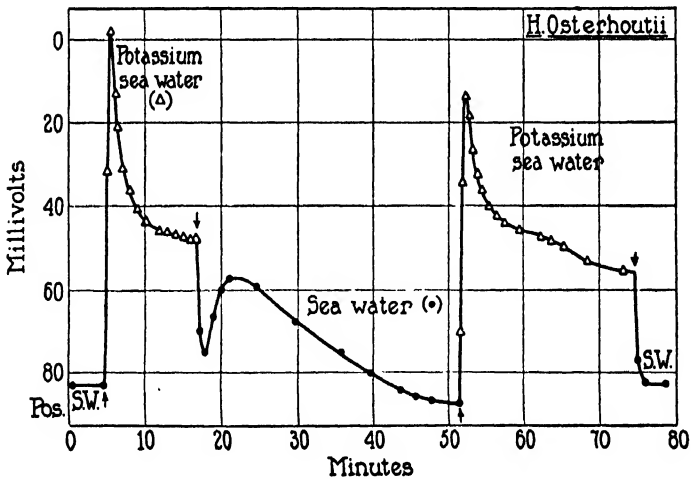


FIG. 8. Potential difference across the protoplasm of *H. Osterhoutii* in normal sea water and in potassium sea water (sodium replaced by potassium).

plasm, it is not to be assumed that  $K^+$  ion has a low mobility therein (or one nearly equal to that of the anion). On the contrary, if KCl diffused extremely readily into the protoplasm, so that the latter soon contained nearly as high a concentration of this salt as the sap, the potential across the inner surface at least would be greatly reduced



(cf. Damon<sup>9</sup>). That this may happen is indicated by the behavior when potassium chloride is applied externally, the rapid lowering of potential probably being due to the diffusion potential, the recovery to the disappearance of the gradient as diffusion proceeds. From the maximum height of the upward cusp, therefore, could be calculated at least a minimum mobility for potassium ion, relative to chloride, and this would have rather a large value, just as in *Valonia*.<sup>9</sup> But except where the curve flattens out well, the author is inclined to believe such calculations rather doubtful in significance, since the height may depend largely upon the speed with which one solution is substituted for another.

A decreased effect of solutions containing potassium chloride is seen when one exposure quickly follows another, with only a short intervening sojourn in sea water (Figs. 4 and 5). This might also indicate that potassium has entered the protoplasm to a considerable extent during the first exposure, and has not rapidly diffused out again.

In order to compare the two species, the same experiments were performed with *H. Osterhoutii*, in which potassium has essentially the same effect. Figs. 7 and 8 show such experiments. There is again a large but temporary decrease of the positive potential, followed by a rise in which the potential is built up to a value about 20 or 30 mv. lower than the original value. Here again we may assume that the cusp represents the diffusion potential of KCl, and the recovery, the diffusion of KCl into the protoplasm (and probably also into the vacuole). The behavior is practically identical in the two species except that potassium ion may have even a higher mobility in *H. Osterhoutii*.

#### *The Interior Alteration of Potassium Content*

We might therefore assume that if the sap of *H. Osterhoutii* could in some manner be increased in potassium content, the potential across the protoplasm would become higher. This assumption has been recently justified by a simple method of vacuolar irrigation, which will be more fully described in another paper dealing with the experimental alteration of cell sap. For present purposes it may be said that it has proved possible to mix the fresh natural sap of *H.*

<sup>9</sup> Damon, E. B., *J. Gen. Physiol.*, 1929-30, 13, 207, and unpublished results.

*Osterhoutii* with an equal volume of 0.6 M KCl solution, thereby raising its potassium content approximately to that of *H. ovalis*. When this mixture is irrigated through the cells of *H. Osterhoutii*, the potential across the protoplasm often rises from 65 mv. to 80 mv. or more, and in some cases has remained at 75 mv. for several days thereafter. This indicates that potassium has much the same effect on the inner surfaces of the protoplasm of both species, and that the higher potential of *H. ovalis* is probably largely due to the higher potassium content of its sap. The reverse experiment, remaining to prove this, would involve the lowering of KCl in the latter species. But to bring this down to the level of *H. Osterhoutii* would require a 30-fold dilution of natural sap with isotonic NaCl, etc., an amount which the cells have so far not been found to endure. Were both species simultaneously available, the fresh sap of *H. Osterhoutii* could probably be substituted in the cells of *H. ovalis* without difficulty, just as that of *Valonia* can be irrigated through *H. Osterhoutii*. It is hoped that this experiment may be performed in the future.

#### CONCLUSION

It should be pointed out that these experiments do not favor the hypothesis that potassium is accumulated more in some plants than in others because its ionic mobility is higher in their protoplasm. In the two species of *Halicystis*, the electrical effects of potassium (hence probably its ionic mobility) are almost identical, or slightly larger in *H. Osterhoutii*. Yet *H. ovalis* accumulates KCl some 30-fold, *H. Osterhoutii* not at all.

For their kind invitation to work at Pacific Grove, and for the use of the facilities of the Jacques Loeb Laboratory, the author is deeply indebted to Dr. W. K. Fisher, Director of the Hopkins Marine Station, and to Dr. G. J. Peirce, executive head of the Department of Botany, Stanford University.

#### SUMMARY

The potential difference across the protoplasm of impaled cells of two American species of *Halicystis* is compared. The mean value for *H. Osterhoutii* is 68.4 mv.; that for *H. ovalis* is 79.7 mv., the sea water being positive to the sap in both.

The higher potential of *H. ovalis* is apparently due to the higher concentration of KCl (0.3 M) in its vacuolar sap. When the KCl content of *H. Osterhoutii* sap (normally 0.01 M or less) is experimentally raised to 0.3 M, the potential rises to values about equal to those in *H. ovalis*.

The external application of solutions high in potassium temporarily lowers the potential of both, probably by the high mobility of  $K^+$  ions. But a large potential is soon regained, representing the characteristic potential of the protoplasm. This is about 20 mv. lower than in sea water.

The accumulation of KCl in the sap of *H. ovalis* is apparently not due to the higher mobility of  $K^+$  ion in its protoplasm, since the electrical effects of potassium are practically identical in *H. Osterhoutii*, where KCl is not accumulated.

## THE KINETICS OF PENETRATION

### IV. DIFFUSION AGAINST A GROWING POTENTIAL GRADIENT IN MODELS

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Cells of *Nitella* in tap water show an outwardly directed E.M.F. of from 50 to 300 millivolts. Previous experiments<sup>1</sup> suggest that this is largely due to potassium ions whose entrance into the vacuole produces an outwardly directed E.M.F. This tends to oppose their further penetration but they nevertheless continue to enter.

Something of this sort seems to happen in certain models recently described.<sup>2</sup> An example is seen in an experiment in which 0.05 M KOH is shaken with a small excess of a mixture of 70 per cent guaiacol and 30 per cent *p*-cresol (which may be called G.C. mixture) which converts most of the KOH to organic salts of potassium. For convenience these organic potassium salts may all be regarded as K-guaiacolate and called KG.<sup>3</sup>

The resulting solution is allowed to flow in a steady stream over a layer of G.C. mixture. We may then say that the KG passes from the outer solution (*A*) through the non-aqueous layer of G.C. mixture (*B*) and penetrates into the inner solution (*C*), which at the start consisted of distilled water in which CO<sub>2</sub> was bubbling. The latter may be regarded as analogous to the sap of *Nitella* and designated as "artificial sap." *B* will then correspond to the non-aqueous protoplasmic layer and *A* to the external solution bathing the *Nitella* cell.

<sup>1</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, **13**, 715; Electrical phenomena in the living cell, in Harvey Lectures, 1929-30, Baltimore, The Williams and Wilkins Co., 1931; *Biol. Rev.*, 1931, **6**, 369.

<sup>2</sup> Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1931-32, **15**, 667.

<sup>3</sup> This involves no serious error as the K-*p*-cresolate seems to behave like the K-guaiacolate.

On reaching *C*, *KG* reacts to form  $\text{KHCO}_3$  which may accumulate until its concentration reaches 0.63 *M*. Hence the osmotic pressure in *C* becomes much greater than in *A*. The energy required to produce this result comes from the reaction of *KG* with  $\text{CO}_2$ . In part this energy finds expression in the form of E.M.F.'s.

In order to measure the E.M.F.'s produced in the model the apparatus shown in Fig. 1 was employed. With this arrangement we may suppose that a thin layer of II, which we may call  $l_o$ , (perhaps only a few molecules thick) will come at once into equilibrium with III and will in consequence have a different composition from the rest of II (which may be called  $l$ ).

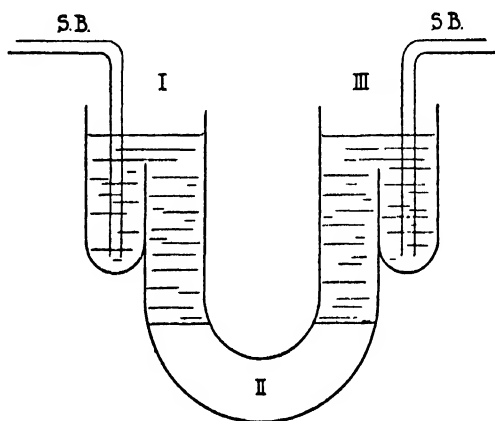
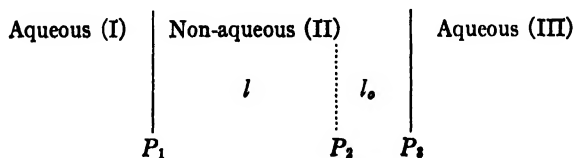


FIG. 1. Aqueous solutions are placed in I and III and a non-aqueous liquid in II. The salt bridges, S.B. (consisting of glass tubes filled with agar made up in saturated KCl solution) were placed at the sides in order that KCl sinking downward might not contaminate the interfaces.

Presumably we have the three potentials shown below, namely,



the phase boundary potentials  $P_1$  and  $P_3$ , and the diffusion potential  $P_2$  in the non-aqueous layer.

Unless otherwise stated the procedure was as follows. The aqueous solution in I (Fig. 1) and the non-aqueous solution in II were shaken together and left in

contact for 24 hours or more before they were placed in the apparatus. The aqueous solution in III was shaken with a small excess of the non-aqueous solution in II, placed in a separatory funnel and freed from the non-aqueous portion which was discarded. Hence when the solutions were placed in the apparatus there was presumably no exchange of ions between I and II, but ions moved from III into II and set up a diffusion potential<sup>4</sup> in II. If there be any diffusion potential in III due to exit of ions from II it must be small.

To imitate electrical conditions in the model near the start<sup>5</sup> of the experiment aqueous solution from *A* (consisting of 0.05 M KG) is placed in I. Non-aqueous solution from *B* (consisting of G.C. mixture containing KG) is placed in II. In III we might place distilled water, but as this gives irregular results we prefer to use 0.001 M  $\text{KHCO}_3$  (which would represent the condition of *C* in Model III<sup>2</sup> a short time after starting the experiment).

This gives a potential of 45 millivolts,<sup>6</sup> I being negative to III in the external circuit (hence *A* is negative to *C* in the model). On bubbling  $\text{CO}_2$  in III (without disturbing the interface unduly) KG in II begins to combine with  $\text{CO}_2$  at the interface to form  $\text{KHCO}_3$  (which is much more soluble in III than in II). Hence the concentration of  $\text{KHCO}_3$  in III increases and that of  $\text{H}^+$  decreases.

This is illustrated by an experiment in which solution from *A* in a model which had been running for some time was placed in I (this consisted<sup>2</sup> of 0.03 M KG + 0.02 M  $\text{KHCO}_3$ ). Solution from *B* was placed in II and 0.0001 M  $\text{KHCO}_3$  in III. At the start the P.D. was 33 millivolts (I being negative to III in the external circuit): this gradually rose to 35 millivolts. Then  $\text{CO}_2$  was bubbled in III and in the course of 70 minutes (shown in Fig. 2) III became negative to I in the external circuit so that positive current tended to flow from III through II into I. Stirring<sup>7</sup> II facilitated this change by bringing more KG to the interface between II and III. In an experiment in which II was stirred occasionally, III became 24 millivolts negative to I (in the external circuit) in the course of 55 minutes. (When III

<sup>4</sup> As a rule the diffusion potentials appear to be much greater in G. C. mixture than in water.

<sup>5</sup> *I.e.*, before much  $\text{KHCO}_3$  entered *A*.

<sup>6</sup> Average of 43, 48, 35, and 54.

<sup>7</sup> This was done in a U-tube having an upright tube in the center through which a stirrer was inserted in II.

is stirred for a moment, it becomes temporarily less negative to I because stirring carries  $\text{KHCO}_3$  away from the interface between II and III.)

These experiments show that in the model the positive current at the start tends to flow from *A* through *B* into *C* but that later this is

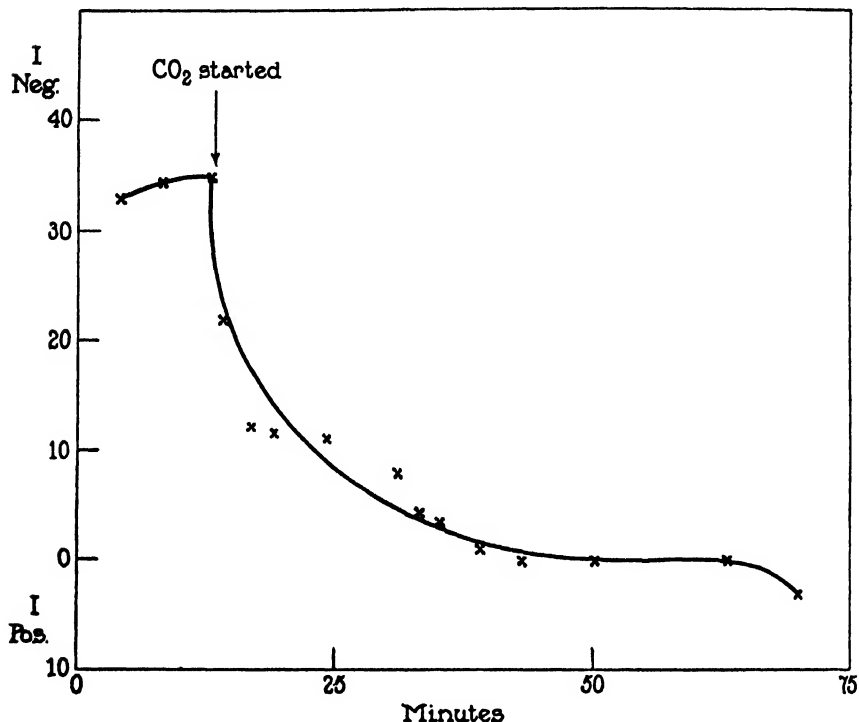


FIG. 2. At the start liquid from *A* in the model is placed in I (Fig. 1) and is 33 millivolts negative (in the external circuit) to 0.001 *M*  $\text{KHCO}_3$  which is placed in III. Liquid from *B* is placed in II. When  $\text{CO}_2$  is bubbled through III it reacts with *KG* in *B* to form  $\text{KHCO}_3$  which dissolves in *C* and reverses the p.d. in the course of 70 minutes.

reversed so that *C* becomes negative to *A* in the external circuit. This negativity continues to increase until the steady state is reached. To ascertain the negativity in the steady state solutions taken from the model in the steady state were placed in the apparatus (solution from *A* was placed in I, from *B* in II, and from *C* in III). This gave 48 millivolts,<sup>8</sup> III being negative to I in the external circuit (hence

<sup>8</sup> This is the average of 5 determinations giving 48, 49, 51, 37, and 53 respectively.

in the model *C* is negative to *A*). Enough KCl<sup>9</sup> was then added to I to make the concentration of potassium equal to that in III and the reading became practically zero<sup>10</sup> despite the fact that I was more alkaline than III (this will be discussed later).

The measurements were made by means of a Compton electrometer (Cambridge Instrument Co.) the use of which was kindly loaned by Dr. Irwin. The shielding and grounding were so carefully carried out that steady readings could be made even with extremely high resistances in the circuit.

Unless the measurements became fairly steady after the first 2 minutes the experiment was rejected (a drift of more than 10 per cent between the 2nd and 10th minute not being acceptable).

It is of interest to know whether the negativity of *C* is due to increase of  $K^+$  or to decrease of  $H^+$ . At the start the activity of  $H^+$  in *C* was between  $10^{-5}$  and  $10^{-6}$  but at the steady state it was  $10^{-7.5}$ ; hence there is about a hundredfold decrease which might account for the change in potential of  $(45 + 48 =) 93$  millivolts were it not for the fact that the activity of  $K^+$  in the meantime increased from zero to  $(0.63)(0.63)^{11} = 0.40$ .

In the presence of so much  $K^+$  it would seem that these variations in  $H^+$  do not cause much change of potential as was indicated by the fact that when the concentration of potassium in I and III was equalized (as just described) the p.d. was practically zero despite the difference in pH value.

In order to test this further a solution of 0.6 M KCl was placed in I and in III: in II was placed G.C. mixture shaken with 0.6 M KCl. As expected this gave zero. Enough HCl was now added to III to make the difference of pH value on the two sides more than 2 pH units; *i.e.*, greater than in the model (in the model the pH value<sup>12</sup> of *A* is 9.1 and that of *C* is 7.5, a difference of 1.6). The reading was still zero or so near it as to be within the limits of experimental error.<sup>13</sup>

<sup>9</sup> KCl has about the same effect on potential as  $KHCO_3$ .

<sup>10</sup> Repeated four times.

<sup>11</sup> The activity coefficient 0.63 is obtained by interpolation from the data of Harned (Harned, H. S., *J. Am. Chem. Soc.*, 1929, 51, 416) on the assumption that it is the same as in a solution of 0.63 M KCl and that the activities of  $K^+$  and  $Cl^-$  in such a solution are equal.

<sup>12</sup> The pH values were determined colorimetrically as described in a former paper.<sup>2</sup>

<sup>13</sup> Repeated three times.



It is therefore clear that in the presence of so much  $K^+$  the observed variations in pH value are negligible as far as effect on potentials is concerned.

We may therefore conclude that in the model the penetration of potassium creates an outwardly directed potential against which it continues to diffuse inward, thereby increasing the outward potential. This continues until the steady state is reached.

It is evident that this potential may be due to phase boundary potential or to diffusion potential.<sup>14</sup> In the latter case we must take into account the inward diffusion of  $KG$ , creating an inwardly directed potential (due to the greater mobility<sup>15</sup> of  $K^+$  as compared with  $G^-$ ) and the outward diffusion of  $KHCO_3$ , producing an outwardly directed potential (due to the greater mobility<sup>15</sup> of  $K^+$  as compared with  $HCO_3^-$ ).

In the case of living cells a similar situation might exist. It has been suggested<sup>1, 2</sup> that potassium may form in the non-aqueous layers of the protoplasm a compound,  $KX$ , which (like  $KG$  in the model) reacts in the sap with an acid  $HA$  to form  $KA$ : subsequently  $HA$  is exchanged for  $HCl$  existing in the external solution. If in the non-aqueous layers of the protoplasm  $KX$  and  $HCl$  pass inward practically undissociated, and  $KCl$  outward, more slowly<sup>16</sup> but with a higher degree of dissociation, the net movement of potassium ions and of chloride ions in the non-aqueous layer will be an outward one so that there might be an outwardly directed diffusion potential<sup>17</sup> due to  $KCl$ . This might account in part for the outwardly directed potential observed in *Nitella* when the sap contains 0.025 M  $KCl$  + 0.025 M  $NaCl$  and<sup>18</sup> the outside

<sup>14</sup> Donnan potential may be ruled out because there are no ions which cannot pass through the non-aqueous phase.<sup>2</sup>

<sup>15</sup> This is indicated by measurements of the concentration effect.

<sup>16</sup> Just as in the model  $KHCO_3$  passes outward more slowly than  $KG$  passes inward. It is supposed that  $KG$  is very little dissociated in the G. C. mixture.<sup>2</sup>

<sup>17</sup> This would be aided by the higher concentration of  $KCl$  and possibly by a greater difference between the mobility of anions and cations in the non-aqueous layers.

<sup>18</sup> Since the protoplasmic surfaces are not identical an organic electrolyte formed between these surfaces may set up an outwardly directed potential which is greater than the corresponding inwardly directed potential. This would account for the outwardly directed potential observed when sap is placed on both sides of the protoplasm (cf. Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927-28, 11, 391).

of the protoplasm is in contact with tap water. (The higher concentration of KCl inside the cell would be due to the energy of metabolism.)

It is of interest to note that if we regard the P.D. as due to diffusion potential we may say that in the model, as well as in *Valonia*, the mobility of the cations appears to be greater than that of the corresponding anions.

#### SUMMARY

In a model consisting of a non-aqueous layer (representing the protoplasm) placed between an inner, more acid, aqueous layer (representing the sap) and an outer, more alkaline, aqueous solution (representing the external solution bathing a living cell) the penetration of potassium creates an outwardly directed potential against which potassium continues to diffuse inward, thereby increasing the outward potential. This continues until the steady state is reached. The potassium sets up less potential in entering than in escaping and the net result is an outwardly directed potential. A similar process appears to take place in certain living cells.



# ON THE MEASURE OF EXCITABILITY

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(Accepted for publication, July 7, 1932)

It was shown by Davis (1923) that the value of chronaxie depends on the nature of the stimulating electrodes. Rushton (1930, 1931) and Lapicque (1931) have confirmed the observation of Lucas (1906-07, 1907-08) that different time-intensity curves can be obtained on the same tissue. The fact that these different curves showed clearly that different measures of chronaxie were obtainable from the same tissue led Lapicque (1931) to adopt his empirical equation,

$$i = a \sqrt{\frac{t + \theta + \sqrt{(t - \theta)^2 + 0.16 \theta^2}}{2 t}}$$

as a criterion for the suitability of any particular time-intensity curve for use as an index of excitability. That is, time-intensity curves which conformed to this equation could be taken as indicative that the method being employed was the proper one for the determination of "true" chronaxie.

Rushton (1932) has recently shown, however, after allowing for possible instrumental errors and for possible inductance in his circuits that time-intensity curves for the frog's sciatic nerve using direct current and Lapicque's electrodes do not conform, even approximately, to Lapicque's equation. He therefore concludes that no use can be made of Lapicque's equation as a criterion.

The purpose of the present paper is to show that Rushton's data in common with that of Lapicque and others do conform to a particular equation but that the use of this fact probably lies in the establishing of experimental rather than theoretical criteria.

The representation of time-intensity curves for various types of electrical stimuli by solutions of the differential equation,

$$\frac{dp}{dt} = KV - kp \quad (1)$$

where  $p$  is the local excitatory process,  $V$  the stimulating voltage, and  $K$  and  $k$  constants, was previously discussed (1932, *a*). Letting the threshold value of  $p$  be a function of the applied voltage of the form  $h \pm \alpha V$  where  $h$  and  $\alpha$  are constants the solution for direct currents is,

$$\log \frac{KV}{KV - k(h \pm \alpha V)} = kt \quad (2)$$

Putting  $V$  equal to the rheobase  $R$ , when  $t$  is great,

$$KR = k(h \pm \alpha R)$$

so that on substituting for  $kh$  in (2),

$$\log \frac{K}{K \mp k\alpha} \times \frac{V}{V - R} = kt \quad (3)$$

or,

$$\log \frac{V}{V - R} = kt + C \quad (4)$$

It is evident on inspection that  $C$  is negative when the threshold is  $h + \alpha V$  and *vice versa*.

In Table I are given the results of applying this formula to Rushton's data. In each case he gave the greatest and least voltage observed. The mean value was used for calculating. It is given in the column *mean V*. He used four separate preparations but obtained two sets of data from each, one with increasing and the other with decreasing voltages. Each set of data was calculated separately as is shown by the table. The unit of time is the second and the constants are calculated to base 10 for convenience. The voltages marked with asterisks in each case were used to determine the constants. The choice of voltages for this purpose was made from graphs with  $V/(V - R)$  on logarithmic scale against time on natural scale. Any two values giving a mean of the linear relation predicted by equation (4)

are suitable. For reasons previously given (1932, *a*) it is necessary to expect linearity only with voltages not very near the rheobase.

It will be seen from the table that the data conform to the equation as well as can be expected and that there are no systematic divergences. Again as with Lapique's data (Blair, 1932, *a*) and as with those considered by Hill (1910) and Lucas (1910) the threshold depends on the voltage in most cases as  $C$  has appreciable magnitude except in set 4 in the second part of which it becomes zero. Sets 3 and 4 were taken near 0°C. The fact that  $C$  is small in these cases compared to sets 1 and 2 which were taken at room temperature may be significant although its magnitude appeared previously (1932, *a*) to be a function of electrodes as well.

In this regard it appeared desirable to investigate the data of Jinaka and Azuma (1923) which were obtained with the pore electrodes of Pratt (1917). They claimed that their data disagreed with Hill's equation and they do. Examination of their papers led to the conclusion, however, that the method they used to calculate their currents was wrong and unfortunately sufficient data were not given to make a recalculation possible. It is assumed therefore that their results have no significance in their present form.

It was previously shown (1932, *a*) and can be readily seen from equation (4) that chronaxie is given by,

$$\tau = \frac{1}{k} (\log 2 \pm C)$$

and is therefore a function of  $C$  as well as of  $k$ . The question arises as to what extent the different chronaxies on the same tissue obtained by different methods are conditioned by changes in  $k$  and to what extent by changes in  $C$ . The importance of this is obvious since chronaxie can only have meaning as a function of variables which are, in turn, functions of those properties of the tissue which govern the rate of excitation.  $k$  itself is of course a direct measure of excitability according to the conception of Keith Lucas (1910) in the sense that it measures the rate of decay of the excitatory process. The quantity  $\log \theta$  derived from Hill's formula and used by Keith Lucas is in fact numerically equal to  $k$  but opposite in sign. There are practical advantages, however, in the use of chronaxie if the experimental condi-

TABLE I

Time	1a			1b		
	Volts	Mean $V$	Calc. $V$	Volts	Mean $V$	Calc. $V$
sec.						
$\infty$	14-13.4	13.7	13.7	13.4-13	13.2	13.2
0.000175	31-30	30.5	33.0	30-29	29.5	30.6
0.00011	47-45	46.0*	46.0	45-43	44.0	43.3
0.00007	65-62	63.5	64.6	63-60	61.5*	61.5
0.000032	115-110	112.5*	112.5	115-110	112.5*	112.5
0.000012	>140		194			213.2
	$k = 1232; C = 0.0171$			$k = 1332; C = 0.0116$		
	2a			2b		
	Volts	Mean $V$	Calc. $V$	Volts	Mean $V$	Calc. $V$
$\infty$	5.0-4.8	4.9	4.9	5.2-4.8	5.0	5.0
0.000295	11.2-10.6	10.9	10.4	11.7-11.2	11.45	10.1
0.000175	14.5-14.0	14.25	15.2	15.0-14.5	14.75*	14.75
0.000106	23-22	22.5*	22.5	22-21	21.5	21.9
0.00007	32-30	31.0	31.2	31-29	30	30.6
0.000035	55-50	52.5*	52.5	55-50	52.5*	52.5
	$k = 901; C = 0.0110$			$k = 974; C = 0.0093$		
	3a			3b		
	Volts	Mean $V$	Calc. $V$	Volts	Mean $V$	Calc. $V$
$\infty$	5.4-5.0	5.2	5.2	6.0-5.6	5.8	5.8
0.00098	13.5-13.0	13.25	13.6	14.0-13.5	13.75	13.6
0.00050	23-22	22.5	23.4	23.5-22.5	23.0	23.2
0.00031	36-34	35.0*	35.0	36-34	35.0*	35.0
0.000195	53-50	51.5	51.6	55-52	53.5	52.6
0.00012	83-78	80.5	77.4	83-79	81.0	80.1
0.00008	107-102	104.5*	104.5	115-110	112.5*	112.5
0.000065	>132		123.4	132		134.7
	$k = 207; C = 0.0055$			$k = 243; C = 0.0034$		
	4a			4b		
	Volts	Mean $V$	Calc. $V$	Volts	Mean $V$	Calc. $V$
$\infty$	10.5-10	10.25	10.25	10.5-10	10.25	10.25
0.00082	19-18	18.5	18.6	19-18	18.5	18.5
0.00050	27-26	26.5*	26.5	28-27	27.5	26.4
0.00031	40-38	39.0	38.9	40-38	39.0*	39
0.000195	62-59	60.5	58.4	60-57	58.5	58.6
0.00012	92-88	90*	90	94-90	92.0*	92.0
0.00008	>130		130.8	>130		138.3
	$k = 421; C = 0.0017$			$k = 427; C = 0.0001 = 0$		

\* The mean voltages marked with asterisks were used to calculate  $k$  and  $C$ , and these constants were used in equation (4) to obtain the calculated voltages (Calc.  $V$ ).

tions can be controlled as it requires only one measurement in addition to the rheobase while the evaluation of  $k$  requires at least two. The situation is not promising, however, as can be seen from the following examples. The largest  $C$  with Rushton's data is 0.0171. In this case chronaxie is proportional to  $\log 2 - C$ ; *i.e.*, to 0.284 while it should be proportional to  $\log 2 = 0.301$ . This is not a great divergence but of the data given in Lapicque's book which were previously considered (1932, *a*)  $C$  was frequently about 0.06 which would make chronaxie proportional to 0.24. In one case  $C$  was 0.128 making it proportional to 0.173 while in Lapicque's later work (1931) the  $C$ 's were sometimes even greater than 0.15 which would make chronaxie at least 100 per cent in error as a measure of excitability on a common basis. Since  $C$  can be either positive or negative as was previously shown (1932, *a*) it is quite evident that chronaxies varying by more than 100 per cent may be obtained on the same tissue in the same state of excitability by virtue of variations of  $C$  with different conditions.

Whether or not the variations of chronaxie with interelectrode distances are due to variations of  $C$  or  $k$  or both cannot be decided with available data. Nor does the extensive work of Rushton (1927) on the variation of threshold with the separation of the electrodes throw any light on the matter. The experimental problem is to obtain time-intensity curves as functions of the interelectrode distances, and of the types of electrodes.

Further, this problem is of great importance in that it will show the dependence, if any, of  $k$  on the positions of the electrodes for it appears quite improbable that the whole burden of the variations of chronaxie with the method of its derivation can be laid upon  $C$  alone. It was shown in discussing Lapicque's recent work that values of  $k$  varying from units to hundreds were obtainable on the same tissue. There seem to be but two possible explanations: the classical one on the basis of the tissue having different excitabilities and one on the basis of  $k$  being a function of the mode of stimulation as well as of the properties of the tissue. In the latter event it would appear quite hopeless to expect ever to measure the excitability proper for even though a method could be found to give consistent results there would be no way of determining from the time-intensity curves themselves whether a real or pseudoexcitability was being measured. The only feature of



the problem which indicates that a real measure of excitability is possible is the fact that a standard technique such as Lapicque's has led to results which classify different tissues into the proper general order. In addition, which is more important, it has led to correlations between chronaxie and such other phenomena considered functions of excitability as the velocity of propagation of the impulse.

It is scarcely possible to avoid adopting the view that the ultimate meaning of excitability can only be in terms of some type of measurement. The requirements of the measure are just that it should be consistent with any other measure which may be a criterion of the same thing. The solution of the problem then from the present point of view depends on whether there can be found by experiment conditions applicable to all tissues which will give consistent and comparable values of  $k$ . If this is found possible it will then be sufficient to define the excitability as  $k$  or as some function of  $k$  providing the results so obtained appear to properly measure the attributes included under the term excitability.

The fact that it seems possible at present that  $k$  may have many different values for the same excitability provides a real difficulty but does not, however, deprive it of its value as a criterion providing that some limiting conditions can be reached in a way analogously, for example, to that by which the rheobase approaches constancy as the interpolar length is increased. A further difficulty may appear, however, in establishing the same condition for different tissues so that the  $k$  of one will be comparable with that of another.

### *The Meaning of $k$*

Since the elimination of  $C$  will probably not be possible except under very particular conditions and since as a consequence it may be necessary to use  $k$  rather than chronaxie as a measure of excitability it will be of interest to consider the meaning of  $k$  in reference to equations (1) and (4).

By equation (1) as was previously indicated,  $k$  is the rate of return to normal per unit of state of excitation.

From equation (4) it is evident that  $k$  is the slope of the graphic representation of a time-intensity curve when  $\log(V/V - R)$  is plotted against time as abscissa. Such a representation would provide a con-

venient picture of comparative excitabilities for, if any sets of curves from different tissues were appropriately shifted so as to pass through the origin, the ordinates for any particular value of the time would be proportional to the respective  $k$ 's of the corresponding tissues.

Putting  $V = nR$  where  $n$  is a number, *i.e.* expressing intensity in rheobases instead of volts or amperes, equation (4) becomes,

$$\log \frac{n}{n-1} = kt$$

Differentiating,

$$\frac{dn}{dt} = n(1-n)k \quad (5)$$

In particular when  $n = 2$ , *i.e.* when  $t =$  chronaxie,  $k$  is numerically equal to  $\frac{1}{2}$  the tangent to the time-intensity curve. No practical use of these relations is probable but they show that the shape of the time-intensity curve on a scale of rheobases is a function of  $k$  alone. In other words, equation (5) is a formal proof that  $k$  and  $k$  only is a factor which expresses the variations of excitabilities as measured by the time-intensity relations.

The extent to which the other constants  $K$  and  $\alpha$  may be evaluated requires consideration in regard to experimental investigations of  $C$ . Taking the case when  $C$  is positive in (4), *i.e.* when  $\log K/(K \pm k\alpha)$  is negative in (3), *i.e.* when  $C = \log (K + k\alpha)/K$  it is evident that from experimental data there are derivable the relations,

$$\frac{K + k\alpha}{K} = C' \text{ where } \log C' = C.$$

This gives on division by  $K$

$$\frac{k\alpha}{K} = C' - 1$$

so that since  $k$  may be determined separately the ratio  $\alpha:K$  is obtainable. From the rheobase conditions  $KR = kh \pm \alpha R$  and the ratio  $\alpha:K$  the ratio  $h:K$  can be calculated and these ratios can be studied as functions of experimental conditions.

The discussion thus far has been based on the assumption that equation (4) is the proper representation of the time-intensity curve, but in view of the possibility that some other equation may be found to represent the data equally well the question arises as to whether chronaxie, since by definition it is quite independent of the shape of the curve, would not be a better measure of excitability than  $k$ . The answer to this is that the whole concept of comparative excitabilities in terms of chronaxies is derived from the assumption that all the time-intensity curves when expressed in the proper units give identical equations. Lapicque (1926, p. 225, footnote) gives the following argument. Let  $\tau_1, \tau_2$  respectively be the time constants for two excitabilities. Then if  $i$  represents the currents and  $a_1$  and  $a_2$  the respective rheobases,

$$\frac{i}{a_1} = f\left(\frac{t}{\tau_1}\right) \quad \text{and} \quad \frac{i}{a_2} = f\left(\frac{t}{\tau_2}\right)$$

*i.e.*, the currents in rheobases are functions of the time in terms of  $\tau$ . If by experiment the times  $t_1$  and  $t_2$  are determined for the condition

$$\frac{i}{a_1} = \frac{i}{a_2}$$

which is the condition for determining chronaxies,

$$f\left(\frac{t_1}{\tau_1}\right) = f\left(\frac{t_2}{\tau_2}\right) \quad \text{or} \quad \frac{t_1}{\tau_1} = \frac{t_2}{\tau_2} \quad (6)$$

*i.e.*, the time constants are proportional to the times required to excite. The validity of this argument depends entirely on the assumption that the two functions concerned are precisely the same. Certainly there does not exist as yet any such set of functions which represent the data adequately. There is therefore no justification at present for the use of chronaxie.

Lapicque's condition does not, however, have to be fulfilled except for making chronaxie valid. It is sufficient for the existence of a time constant which can be used to measure excitability that the conditions of equation (5) should be fulfilled. These conditions are not so restricting as those of Lapicque for chronaxie. With equation (4), for example, as has already been pointed out from experimental con-

siderations the chronaxie condition (Equation 6) requires the identity of the constants  $C$  which is unnecessary when use is being made of  $k$ . If, however, the constancy of  $C$  can be attained experimentally the argument leading to equation (6) shows that the use of chronaxie as a measure of excitability will give just as consistent a scale as  $k$  if the relative ease of its determination makes it preferable for ordinary use.

It may clarify the problem to discuss the conditions with reference to equation (4). According to equation (4) all time-intensity curves can be put in the form,

$$\log \frac{n}{n-1} = T + C \quad (7)$$

where  $n$  is the voltage in rheobases and  $T$  the time in units of  $1/k$ , *i.e.* in the natural tissue units, and  $C$  is as usual. The meaning of this is that all time-intensity curves will be congruent on these scales except for the variable displacement  $C$ . In particular if  $\log n/(n-1)$  is plotted against  $T$  the basic curve is a straight line through the origin whose slope is unity and the plot of any time-intensity data on the same scale will be parallel to this and at a distance  $C$  from it.

Lapicque's condition which is obeyed only when  $C$  in equation (7) is the same for all tissues requires in general that,

$$f(n) = T$$

where  $T$  is measured in chronaxies. If such a function is ever discovered all time-intensity curves on these scales will be congruent without displacements.

The situation may be summed up as follows: chronaxie is no longer valid as a measure of excitability from the point of view of equation (4) since it is a function of the quantity  $C$  which depends on experimental conditions as well as on the time constant  $k$ . The existing data neither provide a means of determining the factors involved in the variations of  $C$  nor indicate how successfully they can be controlled so that the solution of the problem requires a thorough experimental investigation of the time-intensity relations. The data so obtained will also be useful in determining whether  $k$  itself is a function of the experimental method as well as of the properties of the tissue. If it is a function of the method the only hope of obtaining a quantitative scale

of excitabilities is in the determination of standard limiting experimental conditions applicable to all tissues. The criteria for these conditions will for the present probably have to depend on the determination of situations where the experimental variables no longer change or change only very slowly with alterations of the conditions. Eventually they should enable consistent correlations to be established between different phenomena included under the concept of excitability in order to inspire confidence in their validity. Any conclusions drawn from chronaxie measurements, except perhaps those obtained by the same method, must be looked upon with considerable suspicion at present for whether or not equation (4) is the true time-intensity relation it shows that a neglect of the possible effect of boundary conditions is dangerous when drawing conclusions from the time-intensity curve about the properties of the tissue from which it is obtained. Whether or not equation (4) is the true representation it can be used equally well as a basis for research, for since it fits the existing data within the experimental error for all values of durations and voltages its constants must be simply related to those of the true representations except in the unlikely event that the existing data are not representative.

#### SUMMARY

Recent time-intensity data by Rushton (1932) on the sciatic nerve of the frog are shown to provide additional support to the writer's suggestion (1932, *a*) that integrals of the equation

$$\frac{dp}{dt} = KV - kp$$

where  $V$  is the applied voltage,  $p$  is the local excitatory process and  $K$  and  $k$  are constants adequately represent the just effective direct current stimuli when the threshold value of  $p$  is made a linear function of the voltage of the form  $h \pm \alpha V$  where  $h$  and  $\alpha$  are constants.

The measurement of excitability is discussed and it is shown that the criteria for "true" measurements are not likely to be found by the agreement of the data, with canonical time-intensity functions as suggested by Lapicque (1931) but rather in the establishing of standard experimental conditions. These conditions may permit the use of

chronaxie as a measure of excitability, but it seems more likely that the constant  $k$  of the above equation will have to be adopted. There is sufficient evidence to cast considerable doubt on the validity of any conclusions drawn from the existing measurements of chronaxie although those derived through a particular technique may be valid. The problem requires a thorough experimental investigation in terms of integrals of the above equation.

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# ON THE EXCITATION OF TISSUE BY MEANS OF CONDENSER DISCHARGES

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In a previous discussion (1932, *b*) the method of obtaining a general equation for condenser discharge stimuli was given, but the possible dependence of the threshold on the applied voltage was neglected. There is so much use made of the condenser in stimulating that it appears desirable to consider the general equations more fully, since they may be given comparatively simple forms even when the threshold is made a linear function of the voltage.

Using the same notation as before (1932, *b*), the differential equation of the local excitatory process  $p$  with respect to time is given by,

$$\frac{dp}{dt} = \frac{Kq_0 e^{\frac{-t}{cr}}}{c} - kp \quad (1)$$

where  $q_0$  is the initial charge of the condenser,  $c$  its capacity,  $r$  the resistance of the circuit, and  $K$  and  $k$  are constants. The data on direct currents (1932, *a*, *c*) indicate that the upper limit of integration, *i.e.* the liminal threshold value of  $p$ , should be a function of the voltage of the form  $h \pm \alpha V$  where  $V$  is the voltage at the time when the local excitatory process becomes adequate and  $h$  and  $\alpha$  are constants. This condition may be introduced.

An integral of (1) is given by

$$p = \frac{Kq_0 r}{crk - 1} \left\{ e^{\frac{-t}{cr}} - e^{-kt} \right\} \quad (2)$$

but  $p$  must be a maximum when the response occurs if the stimulus is just adequate. Therefore from (1), since  $\frac{dp}{dt} = 0$  for a maximum,

$$kp = \frac{Kq_0}{c} e^{\frac{-t}{cr}} \quad (3)$$



Equating these values of  $p$  from (2) and (3),

$$t = \frac{cr}{crk - 1} \log crk, \quad (4)$$

where  $t$  is the *durée utile* of the discharge.

Putting the threshold  $p = h \pm \alpha V$  in (2), this equation becomes on substituting for  $t$  from (4)

$$h \pm \alpha V = \frac{Kq_0 r}{crk - 1} \left\{ (crk)^{\frac{1}{1-cr k}} - (crk)^{\frac{cr k}{1-cr k}} \right\} \quad (5)$$

where  $V$  is the value of the applied potential at the time the local excitatory process has just become adequate. This potential, however, using the equation of the discharge of the condenser is given by

$$V = \frac{q}{c} = \frac{q_0 e^{-\frac{t}{cr}}}{c} = \frac{q_0}{c} (crk)^{\frac{1}{1-cr k}},$$

when  $t$  is again obtained from (4). Equation (5) may therefore be written

$$h \pm \frac{\alpha q_0}{c} (crk)^{\frac{1}{1-cr k}} = \frac{Kq_0 r}{crk - 1} \left\{ (crk)^{\frac{1}{1-cr k}} - (crk)^{\frac{cr k}{1-cr k}} \right\} \quad (6)$$

But since  $crk (crk)^{\frac{cr k}{1-cr k}} = (crk)^{\frac{1}{1-cr k}}$ , equation (6) becomes on making this substitution,

$$h = q_0 r (crk)^{\frac{cr k}{1-cr k}} \{K \pm k \alpha\} \quad (7)$$

It may sometimes be legitimate to adopt the device of considering  $crk \gg 1$  when the greatest capacity is being used; *i.e.*, in finding the condenser rheobase condition. In this case (7) may be written,

$$kh = \frac{q_0}{c} \{K \pm k \alpha\} = R_c \{K \pm k \alpha\} \quad (8)$$

where  $R_c$  is the rheobase voltage of the condenser.

Substituting the value of  $K \pm k \alpha$  from (8) into (7) and dividing by  $h$ ,

$$\begin{aligned}
 R_c &= q_0 r k (crk)^{\frac{crk}{1-cr k}} \\
 &= V(crk)^{\frac{1}{1-cr k}}
 \end{aligned}
 \tag{9}$$

where  $V$  is the initial voltage  $\frac{q_0}{c}$  of the condenser in any particular case.

Equation (9) should be quite general for condenser discharge stimuli, providing new phenomena, not previously encountered with direct current stimuli, do not exist; and providing also that sufficiently large resistance and capacity have been used to ensure the validity of the assumption that  $crk$  greatly exceeds unity.

It will be much better in general to determine the direct current rheobase and to use the relation (1932, *a*, page 717),

$$kh = R \{K \pm k \alpha\} \tag{10}$$

where  $R$  is the direct current rheobase and the other letters have the same meanings as before. It will probably be essential in determining this rheobase to maintain the same experimental conditions as are used with the condenser stimuli so that the constants will be applicable.

Substituting again in (7) for  $K \pm k \alpha$  from (10),

$$\frac{R}{V} = (crk)^{\frac{1}{1-cr k}} \tag{11}$$

which is the same as (9) except for the rheobase term,  $R$ .

This equation should relate the direct current rheobase with the capacity, voltage, and resistance of the condenser stimulator when it is yielding just adequate stimuli. It may be written,

$$\log \frac{V}{R} = \frac{1}{1-cr k} \log \frac{1}{cr k} \tag{12}$$

or,

$$cr \log \frac{V}{R} = \frac{1}{k} \left\{ \log \frac{V}{R} + \log cr \right\} + \frac{\log k}{k} \tag{13}$$

so that the left hand member is a linear function of the right and the line obtained by plotting these derivations from the data should, if

the relation is exactly obeyed, have a slope numerically equal to  $1/k$  and should intercept the axis of the bracketed member at the point  $-\log k$ . If the relation is not exactly obeyed on account of experimental variations, the divergence from linearity may be great at certain stages for small variations and also the mean linear relation obtained by drawing a line most nearly through the points is not necessarily the best approximation. The method of equation (13), however, can be used fairly easily when the measurements are nearly exact.

It is probably seldom safe to use two equations of type (12) and to subtract them to eliminate  $\log k$ . This method was previously used (1932, *b*, Table I) with *durée utile* data. In doing this it has to be assumed that the  $k$  of  $\log k$  and the other  $k$  are exactly equal, otherwise  $\log k$  is an arbitrary constant which has to be adjusted by trial until the two values of  $k$  are equal. This was not noticed when deriving the table referred to above, so that only those experimental *durées utiles* which were not used in calculating can be compared with the theoretical values.

It will be shown later that  $k$  can be found very easily when the proper data are available, but for the present it will be necessary to use the method of equation (13), which is very tedious.

A matter of the greatest theoretical importance is the determination of whether the factor  $k$  for the condenser discharge, *i.e.*  $k$  of equation (12), is the same as the factor  $k$  for direct current stimulation of the same tissue at the same time. The use of the direct current rheobase in equation (12) implies, of course, the equality of these constants, but it is desirable that it should also be shown directly. Lapique (1907) obtained a set of data on the sciatic-gastrocnemius preparation of the frog suitable for testing this relation. These data will now be considered.

Part (*a*) of Table I gives the result of stimulating with direct currents. The two observed voltages marked with asterisks were used to determine the constants  $k$  and  $C$  of the direct current equation (1932, *a*),

$$\log \frac{V}{V - R} = kt + C \quad (14)$$

where  $V$  is the least voltage required to excite when applied for time  $t$ , and  $R$  is the rheobase. These data conform to equation (14) quite well, as can be seen from the table of calculated voltages.

Part (b) of Table I gives the data for stimulation of the same preparation by means of condenser discharges. The numbers  $V_{obs.}$  are the least voltages required to excite when the capacities  $c$  of the condenser and the resistance  $r$  of the circuit, the latter being assumed to be constant, are such that their products give the numbers  $cr$ .  $R$  is the direct current rheobase taken from part (a) of the table.

If now equation (14) correctly represents direct current stimulation and equation (12) condenser discharge stimulation, the quantity  $k = 2428$ , taken from part (a) of Table I, when substituted in the right

TABLE I

(a) Direct current. $k = 1054.3$ or $2428.1$ to base $e$					
$t$ sec. ....	0.00063	0.00093	0.00155	0.00185	
$V$ obs. ....	*0.170	0.130	*0.109	0.1045	
$V$ cal. ....	0.170	0.1284	0.109	0.1045	
(b) Condenser discharges. $r = 70,000$ ; $k = 2428.1$					
$cr$ .....	0.070	0.007	0.0035	0.0014	0.0007
$V$ obs. ....	0.106	0.123	0.140	0.185	0.255
$V$ cal. ....	0.1077	0.1247	0.1391	0.1739	0.223
$R$ obs. ....	0.1045				
$R$ cal. ....	0.103	0.103	0.105	0.111	0.119
$V/R$ obs. ....	1.014	1.177	1.340	1.770	2.440
$V/R$ cal. ....	1.031	1.194	1.331	1.665	2.134
<i>Durée</i> obs. ....	0.00181	0.00154	0.00135	0.00093	0.00063
<i>Durée</i> cal. ....	0.00210	0.00135	0.00099	0.00071	0.00053

Units—volts, ohms, farads, seconds.

\* These two observed voltages were used to determine the constants  $k$  and  $C$  of the direct current equation (1932, a).

hand member of equation (12) should give the left hand as obtained from the measured values of  $V$  and  $R$ ; *i.e.*, the observed values of  $V/R$  should equal the values obtained from solving the right hand of (12). The columns  $V/R_{obs.}$  and  $V/R_{cal.}$  respectively give this comparison. It will be seen that the agreement is not bad.

Using equation (12) again and assuming the measured voltage,  $cr$ , and the  $k$  from part (a) of the table to be correct, the rheobase may be calculated giving the numbers  $R_{cal.}$  It will be seen that these tend systematically to become too large for the greatest voltages but the

mean is not far from the measured value. Considering that Lapicque expressed the fear that he had been unable to avoid appreciable self-induction in the condenser circuit, and considering the possible variations of the excitability  $k$  and of the resistance of the circuit during the experiment, these agreements can be taken as indicating the validity of both equations (12) and (14).

Combining equations (4) and (12) it will be seen that the *durée utile* is given by,

$$t = cr \log \frac{V}{R} = 2.303 \text{ } cr \log_{10} \frac{V}{R} \quad (15)$$

In the table are given the observed values and the values obtained using  $V/R_{cal.}$  The calculated *durées utiles* depend therefore on the measurement  $cr$  and the direct current  $k$ . The agreements are not very good, but as was previously indicated (1932, *b*, Fig. 1) the *durée utile* is subject to large errors in measurement because the curve of the response process  $p$  is relatively flat near its maximum. In making these determinations the measured minimal stimulus will be, in general somewhat more than adequate, so that the local excitatory process will usually attain its threshold value somewhat sooner than it tends to reach its maximum. For this reason the measured *durée utile* will probably be smaller in most cases than the theoretical value. In the present case, Table I, the measured values are usually larger than the theoretical but this may be due to other causes.

The direct current rheobase has seldom been determined in conjunction with complete condenser discharge curves. Two cases on nerve-muscle preparations of *Helix* have been given by L. and M. Lapicque (Lapicque, 1926). Part of these same data concerning the *durées utiles* was previously considered (1932, *b*) but the method was faulty as was previously remarked. These data are given in Table II. In these cases  $k$  had to be determined from the data themselves. The method used was that suggested by equation (13); *i.e.*,  $cr \log V/R$  was plotted against  $\log V/R + \log cr$ . The result in the case of the first set is given in Fig. 1. If the data conform to equation (13) it should be possible to draw very nearly through these points a line whose slope equals the logarithm of its intercept on the horizontal axis. The straight line drawn in the figure has a slope  $1/k = 1/34.25$

and its intercept =  $-1.5347 = \log 1/34.25$ . It will be seen that the points conform to this line fairly well. In general the upper points on such a graph, particularly when  $V$  is close to  $R$ , are very sensitive to changes of  $V$  or  $R$  so that they may frequently diverge greatly from the linear relation. If this divergence is assumed to be due to an error in the rheobase a means is provided for estimating the proper value of the rheobase, for, since the lower points are not much changed

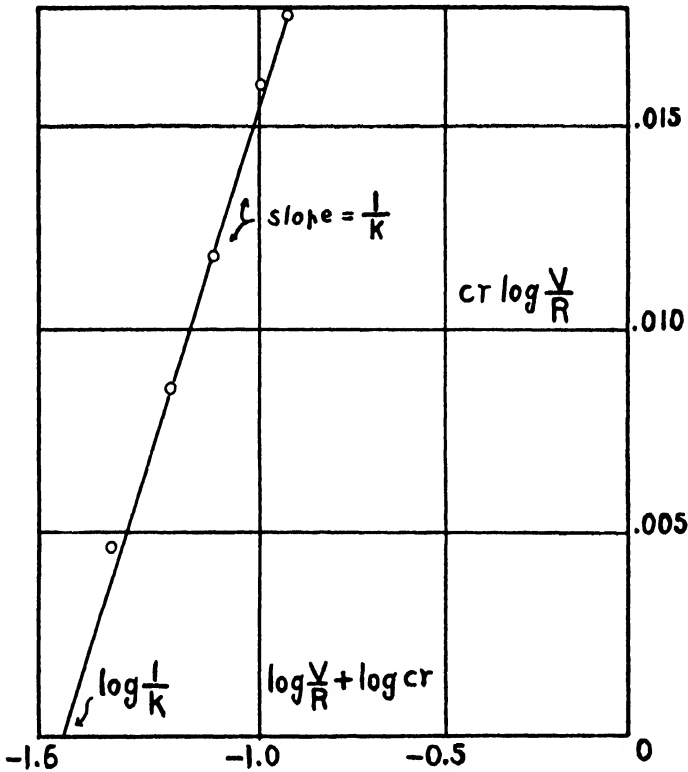


FIG. 1

by small changes of the rheobase the line determined by them is approximately correct and the proper rheobase can be assumed to be that one which brings the upper points close to this line. This method is particularly useful when the rheobase has not been measured at all.

For the first experiment in Table II,  $\log V/R$  obs. is derived from the measured values of  $V$  and  $R$ . The quantity  $\log V/R$  cal. is equal to  $1/(1 - crk) \log 1/crk$  calculated from  $cr$  and  $k$ . The numbers  $R$  cal.

result from assuming the theoretical value of  $V/R$  and the measured  $V$  to be correct. Similarly  $V$  cal. is obtained assuming the theoretical  $V/R$  and the measured  $R$  to be correct. The *durées utiles* are calculated from the relation

$$t = cr \log \frac{V}{R}$$

where  $V/R$  is the calculated value.

In the second experiment of Table II the measured value of  $R$  appeared from a plot like Fig. 1 to be too small. A somewhat larger

TABLE II

<i>Helix</i> , nerve-muscle (Lapicque, 1926, p. 122). $r = 56,500$ ; $k = 34.25$					
$cr$ .....	0.059	0.0395	0.0225	0.014	0.0056
$V$ obs.....	4.0	5.1	6.7	8.2	14.0
$V$ cal.....	3.98	4.72	6.23	8.2	15.43
$R$ obs.....	2.0				
Log $V/R$ obs.....	0.3010	0.4065	0.5250	0.6128	0.8451
Log $V/R$ cal.....	0.2996	0.3730	0.4943	0.6127	0.8876
$R$ cal.....	2.006	2.16	2.15	2.001	1.814
<i>Durée</i> obs.....	0.042	0.026	0.016	0.012	0.0065
<i>Durée</i> cal.....	0.041	0.037	0.027	0.020	0.011

<i>Helix</i> , nerve-muscle (Lapicque, 1926, p. 121). $r = 50,000$ approximately; $k = 59.6$				
$cr$ .....	0.050	0.025	0.015	0.005
$V$ obs.....	6.8	9.0	11.5	18.0
$V$ cal.....	6.44	9.03	10.69	20.8
$R$ obs.....	3.4 assumed for calculation 3.7			
$R$ cal.....	3.9	3.4	4.0	3.2
<i>Durée</i> obs.....	0.038	0.013	0.009	0.0043
<i>Durée</i> cal.....	0.028	0.022	0.017	0.0075
<i>Durée</i> cal.....	0.035	0.024	0.018	0.0083

Units—volts, ohms, farads, seconds.

value 3.7 instead of 3.4 was therefore assumed. It is close to the mean of  $R$  cal. The lower values of the *durées utiles* were obtained from the observed values of  $V$  and  $R$  while the upper were obtained from the calculated.

It will be seen, considering the difficulties of determining the best value of  $k$ , that these data agree with the theory as well as is generally expected in this type of work.

TABLE III

(Cluzet, 1904, p. 210), frog's sciatic. $r = 660,000$ ; $k = 1567$				(Waller, 1900, Exp. 1, p. 212), nerve-muscle preparation, frog? $r = 150,000$ ; $k = 1060$			
$cr$	$V$ obs.	$V$ cal.	$R$ cal.	$cr$	$V$ obs.	$V$ cal.	$R$ cal.
0.66	0.32	0.312	0.31	0.012	0.09	0.095	0.077
0.066	0.32	0.324		0.0012	0.18	0.196	
0.0066	0.40	0.398		0.000375	0.36	0.356	
0.00066	1.00	0.829		0.00015	0.72	0.686	
0.000066	3.80	3.90		0.00006	1.44	1.458	
(Lucas, 1906, Exp. 1, p. 378), frog sartorius. $r = 13,200$ ; $k = 385$				(Cybulski and Zanietowski, 1894, Exp. 1, p. 81), frog's sciatic. $r = 25,200$ ; $k = 2850$			
$cr$	$V$ obs.	$V$ cal.	$R$ cal.	$cr$	$V$ obs.	$V$ cal.	$R$ cal.
0.033	0.448	0.520	0.419	0.0126	0.095	0.099	0.090
0.0133	0.672	0.622		0.00237	0.125	0.125	
0.00726	0.896	0.744		0.000504	0.195	0.206	
0.00307	1.12	1.05		0.000252	0.274	0.292	
0.000792	2.24	2.32		0.000126	0.468	0.445	
0.000294	4.48	4.94		0.0000139	10.1	2.60	
0.000198	6.72	6.79					
0.000145	8.96	8.80					
0.000106	11.20	11.80					
(Weiss, 1901, p. 431), frog's sciatic. $r = 802,000$ ; $k = 1247$				(Hermann, 1906, Exp. 13, p. 546), frog's tibialis. $r = 125,650$ ; $k = 1538$			
$cr$	$V$ obs.	$V$ cal.	$R$ cal.	$cr$	$V$ obs.	$V$ cal.	$R$ cal.
0.1604	0.68	0.67	0.655	0.1257	0.3087	0.3020	0.2932
0.01604	0.93	0.77		0.0628	0.3087	0.3077	
0.00802	0.98	0.85		0.0251	0.3087	0.323	
0.00401	1.10	0.98		0.0126	0.3368	0.343	
0.001604	1.31	1.31		0.00628	0.3740	0.381	
0.000802	1.79	1.79		0.00251	0.4615	0.469	
0.000561	2.08	2.18		0.00126	0.6231	0.595	
0.00040	2.51	2.67		0.000628	0.8287	0.824	
0.000321	2.99	3.05		0.000251	1.4286	1.380	
0.000241	3.94	3.72		0.000126	2.2222	2.243	

Units—volts, ohms, farads, seconds.

In Table III are given sets of data by various workers which can probably be taken as representative. In none of these cases was a rheobase measured so that a value had to be determined. This is



equivalent to assuming two arbitrary constants in equations (11) or (12) except that the one,  $R$ , must have a likely value; *i.e.*, it must in general be just somewhat less than the least value of  $V$ . The rheobase  $R$  cal. was usually obtained by assuming a probable value and testing on a plot like Fig. 1. If the ordinates of the points for voltages near the rheobase are too low for a linear relation they indicate that the chosen rheobase is too large and *vice versa*. The remaining calculations were then made in the same way as those for Table II.

In all these cases it will be seen that the agreement between the observed and calculated voltages is fairly close, except for the greatest voltage of Cybulski and Zanietowski. This case, in which the voltage is about one hundred times the rheobase, may indicate that equation (12) is not valid for very high voltages but it cannot be given much significance since the other data do not show the same tendency and since a small change of capacity involves a large change in voltage at this part of the  $V-cr$  curve. In general with these data such divergences as exist are not systematic. The agreement of the data and the theory is not as good as with direct current data in general, but neither are the experimental factors so easily controlled. In particular the condenser may cause trouble either on account of the nature of the dielectric or on account of leakage. Also the data include the three measurements  $V$ ,  $c$ , and  $r$  each of which may be somewhat in error, and finally the resistance used may not be sufficiently non-inductive. This latter circumstance would affect equation (12) not only through a distortion of the curve of the growth of the local excitatory process but also because the theoretical equation of the discharge of the condenser will no longer give the voltage existing at the time of the response. Inductance with direct current stimuli on the other hand will not affect the latter factor.

### *The Determination of $k$*

Putting equations (11) or (12) in the form,

$$\frac{V}{R} = (crk)^{\frac{1}{crk-1}} \quad (16)$$

it will be seen that when  $crk = 2$ ,  $V = 2R$ . This relation which was previously deduced (1932, *b*) is still true here when the threshold

is made a function of the voltage. If then data are obtained giving the direct current rheobase and the value of  $cr$  for twice the rheobase,  $k$  may be obtained directly from the relation,

$$k = \frac{2}{cr}$$

This method might have been used in the first set of data in Table II. In this case when  $V \text{ obs.} = 4 = 2R \text{ obs.}$ ,  $cr = 0.059$ .  $k$  should therefore be equal to  $2/0.059 = 33.9$ . The value obtained graphically, 34.25 is approximately the same.

It may also be possible when sufficiently large capacities are available to use the condenser rheobase in the same way in many cases. An example is given in Table IV with data by Waller on the ulnar nerve of man.  $k$  in this case was determined by assuming 10 the least value

TABLE IV

(Waller, 1900, Exp. 7, p. 218), ulnar nerve of man. $r = 12,000$ ; $k = 1190$							
$cr$ .....	0.0096	0.00168	0.00066	0.00042	0.00030	0.000216	0.000180
$V \text{ obs.}$ .....	10	20	30	40	50	60	70
$V \text{ cal.}$ .....	10.1	20.0	30.8	40.0	49.4	62.2	70.6
$R$ .....	10.0						

Units—volts, ohms, farads.

of  $V \text{ obs.}$  to be the rheobase. When  $V \text{ obs.} = 20$ ,  $cr = 0.00168$ . Therefore  $k = 2/0.00168 = 1190$ . It will be seen that the use of this value of  $k$  gives  $V \text{ cal.}$  very close to the measured values, the largest divergence being less than 4 per cent. In general, however, it will be necessary to use the direct current rheobase, but the determination of  $k$  in this way is still comparatively easy.

### General Considerations

The data discussed here, although not sufficiently general to be entirely conclusive, particularly in regard to the relation of the direct current equation to the condenser equation, indicate strongly the validity of the hypotheses used in the derivation of equation (12) or (16). The data on *durées utiles* do not agree very well with the theo-

retical values, but since the theoretical value of the *durée utile* is implied in equation (12) which fits the data sufficiently well, it seems likely that the disagreement is due to the difficulty of measurement rather than to an erroneous conception.

In regard to the measurement of excitability by means of condenser discharges it will be seen that apart from considerations previously discussed (1932, *c*) involving the possible dependence of the apparent excitability on the method of measuring it, either chronaxie or  $k$  will give a comparable scale. For since chronaxie is taken directly proportional to  $cr$  when  $V = 2R$ , and since  $k$  is inversely proportional to this same  $cr$ , the scale of excitabilities determined by chronaxie will be the inverse of the scale determined by  $k$ . In the condenser case therefore the only advantage in the use of  $k$  rather than chronaxie is that  $k$  is a direct measure while chronaxie is inverse. Since, however, with direct currents (1932, *a*, *c*)  $k$  and chronaxie are not simply related except in particular cases the use of  $k$  is preferable throughout.

Previous discussions in conjunction with the present one indicate that a satisfactory symbolic basis has been provided for at least the principal phenomena of electrical excitation. The simplicity of the hypotheses gives promise that the elucidation of the actual mechanism in physicochemical terms may not be eventually very difficult, and for the present the simplicity of the analysis will allow the correlation of most of the experimental data which may be obtained.

#### SUMMARY

Equations are derived for the capacity-voltage relations for stimulation of tissue by condenser discharges, using the hypothesis that the local excitatory process  $p$  grows under the influence of an applied potential  $V$  according to the equation,

$$\frac{dp}{dt} = KV - kp$$

where  $K$  and  $k$  are constants. It is further assumed that the local excitatory process becomes adequate when it attains a value  $h \pm \alpha V$  where  $h$  and  $\alpha$  are constants and  $V$  is the applied potential at the particular instant that the adequate value is attained. The equations so obtained are applied to the data of several authors on several

types of tissue and the agreements obtained are sufficiently good. It is shown in one case that the direct current equation and the condenser discharge equation each derived from the above bases are consistent when applied to data from the same preparation.

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# THE PHOTOCHEMICAL REACTION IN PHOTOSYNTHESIS

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## I

From the experiments of Warburg and Negelein (1923), we know that the green alga *Chlorella pyrenoidosa* can reduce one molecule of carbon dioxide for each four quanta of light absorbed, when conditions permit maximum efficiency. Chlorophyll is clearly the substance absorbing the light quanta, so we may inquire how much chlorophyll must be present for the reduction of one molecule of carbon dioxide.

In a preceding paper (1932) we have presented evidence that the mechanism involved in the photochemical reaction must undergo a slower reaction, the so called Blackman reaction, before it can again take part in the photochemical reaction. Let us consider a cell in flashing light when the dark periods between flashes are so long that each unit activated in a given flash has time to complete the Blackman reaction before the next flash. Increasing the intensity of the flashes should increase the carbon dioxide reduction per flash until each unit capable of undergoing the photochemical reaction does so once in each flash. We say then that the photochemical reaction is saturated with light. The possibility that any unit will undergo the light reaction more than once in a single flash may be neglected, because the time required for the completion of the dark reaction is about 0.02 sec. at 25°C., while the duration of a light flash is  $10^{-6}$  sec.

We define one unit arbitrarily as the mechanism which must undergo the photochemical reaction to reduce one molecule of carbon dioxide. If we can obtain light flashes of sufficient intensity to saturate the photochemical reaction, then the number of units in a sample of cells will equal the number of carbon dioxide molecules reduced per flash. The

total chlorophyll content of the sample divided by the number of carbon dioxide molecules reduced per flash will give the number of chlorophyll molecules per unit, or per molecule of carbon dioxide. The measurement of this ratio was the objective of the work described in this paper.

## II

### *Methods of Measurement*

Photosynthesis was measured manometrically in the usual way. The cells were suspended in a mixture of 85 parts  $\frac{M}{10}$  potassium bicarbonate and 15 parts  $\frac{M}{10}$  potassium carbonate. The flashing light was obtained by discharging a 1 or  $\frac{1}{2}$  microfarad condenser, charged to about 3000 volts, through a neon tube. The circuit is described by Emerson and Arnold (1932, p. 395). The tube was flashed twelve or twenty-one times a second.

To obtain flashes of sufficient intensity to saturate the photochemical reaction we were obliged to concentrate the light with mirrors. The voltages used on the condenser were already so high that each tube lasted only a short time. A cylindrical mirror was made by splitting a glass tube about 2 cm. in diameter and silvering the outside of one half. This mirror was hung just below the neon tube, and served to concentrate the light on the vessel containing the photosynthesizing cells. The sides and top of the vessel were also silvered, and all silvered surfaces were copper-plated to protect the silver. Using a cell suspension as a photometer, we found that the mirrors increased the light intensity three to four times.

Ordinary incandescent lamps were not adequate to saturate certain samples of cells with continuous light. We obtained very intense continuous light from 100 watt high temperature projection lamps by adjusting silvered watch-glasses of appropriate diameter and curvature so that the images of the filaments fell in the cell suspensions. Even the intensity so obtained was not wholly satisfactory.

The light intensity was varied quantitatively by attaching neutral filters of known percentage transmission to the bottoms of the vessels. These filters are sufficiently non-selective for white light, but we found denser filters could transmit more red light than their indicated values. The filters used in our experiments with neon light were calibrated for red with the spectrophotometer.

The chlorophyll content of the cells had to be determined in absolute units. We are much indebted to Dr. Hans Gaffron for a sample of chlorophyll a + b prepared from *Chlorella*, with which we standardized our measurements. 13.3 mg. of dry chlorophyll, weighed accurately to 0.1 mg., were dissolved in 1 liter of pure methanol. The extinction coefficient of this solution was measured with a König-Martens spectrophotometer, using light of 6598.95 Å from a neon tube. Prior to measurement the usual Nicol ocular mounted in the divided circle was

replaced by the Gauss ocular, which shows the lines of the neon spectrum separately when the collimator slit is small. The telescope was adjusted so that the line 6598.95 Å was about centered in the field. Then the collimator slit was opened until the neighboring lines on either side, 6532.88 and 6678.27 Å, were about to fuse with the center line. Telescope and ocular slit were then adjusted so that only the line 6598.95 Å was visible. This gives strictly monochromatic light of adequate intensity for such chlorophyll solutions as we prepared.

We have used the definition of the extinction coefficient,  $\epsilon$ , given in the *Handbuch der Physik* (1928, p. 189) from the equation

$$I_1 = I \times 10^{-\epsilon d}.$$

This means that  $\epsilon$  is the reciprocal of that thickness of medium which will reduce the light to one-tenth its original intensity.

Our standard chlorophyll solution, 13.3 mg. per liter of methanol, gave a value of 0.634 for  $\epsilon$  at 6598.95 Å. Reduced to 10 mg. chlorophyll per liter,  $\epsilon = 0.476$ .

For determining the chlorophyll content of cells, samples of about 10 c.mm. were used. After being washed in distilled water, boiling water was poured over them. They were allowed to stand in this for 2 minutes. The treatment does not decrease the yield of chlorophyll, and allows quicker completion of the extraction. The cells were then centrifuged out of distilled water, and extracted with methanol until they were white. Extracts were made up to 25 c.cm. in volumetric flasks. The technique of measuring the extinction coefficients was the same as described above for the standard solution.

If  $v$  is the volume in c.mm. of cells extracted,  $m$  the molecular weight of chlorophyll,  $\epsilon$  the standard extinction for 10 mg. of chlorophyll per liter, and  $\epsilon_1$  the coefficient for the sample, then the number of mols of chlorophyll per c.mm. of sample equals:

$$\frac{\epsilon_1}{v \epsilon m} \times \frac{25 \times 10}{1000^2}.$$

For  $m$  we used 906.6, the value given by Willstätter and Stoll (1913, p. 128) for the average molecular weight of a mixture of chlorophyll a + b. Even large changes in the ratio a:b would not alter this value as much as 1 per cent, since the molecular weights of the two chlorophylls differ by only 14 units, according to Willstätter and Stoll.

The chlorophyll concentration per unit volume of cells was varied by growing cultures over different colors of light. It has been mentioned (Emerson and Arnold, 1932) that satisfactory changes in the chlorophyll content of *Chlorella pyrenoidosa* cannot be effected conveniently by the method of lowered iron concentration used for *C. vulgaris* (Emerson, 1929). But cultures of *C. pyrenoidosa* grown over mercury luminous tubes contain large amounts of chlorophyll per unit amount of cells, while similar cultures grown over neon tubes contain about one-fourth as much chlorophyll. Cells grown over 40 watt incandescent lamps develop an intermediate amount of chlorophyll. The chlorophyll concentration produced



appears to depend on the intensity of the light and the age of the culture, as well as on the color of the light. The neon light cultures mature faster than the incandescent light cultures, the mercury cultures much more slowly. All cultures were grown at 20°C.

### III

#### EXPERIMENTAL

The flashing light concentrated by mirrors was barely sufficient to saturate the photochemical reaction, as shown by Fig. 1. Photosyn-

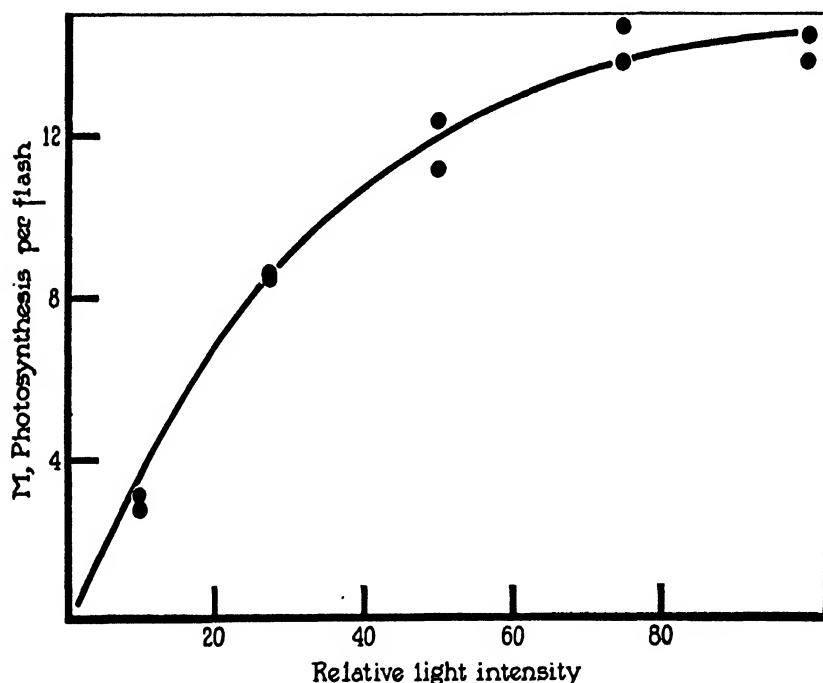


Fig. 1. Relative light intensity plotted against photosynthesis per flash, in arbitrary units. Temperature 25°C.

thesis per flash is plotted against intensity. The shape of the curve shows that higher intensities would probably increase the yield, though the maximum seems to have been nearly attained. This conclusion is in harmony with a possible theoretical explanation of the process which we shall propose in the last section. Table I gives the data for the two experiments incorporated in Fig. 1.

The ratio of chlorophyll content to the maximum height of Curve 1 gives the ratio of carbon dioxide reduction per flash, to amount of

chlorophyll. It is important to know whether this ratio remains constant for different concentrations of chlorophyll, so we have measured maximum photosynthesis and chlorophyll content for a number of cell samples grown so as to have different amounts of chlorophyll. The results, shown in Columns 4 and 10 of Table II, are plotted in Fig. 2. Column 11 of Table II gives the ratio,  $\rho$ , of chlorophyll: photosynthesis per flash at saturation. A straight line is the best fit for

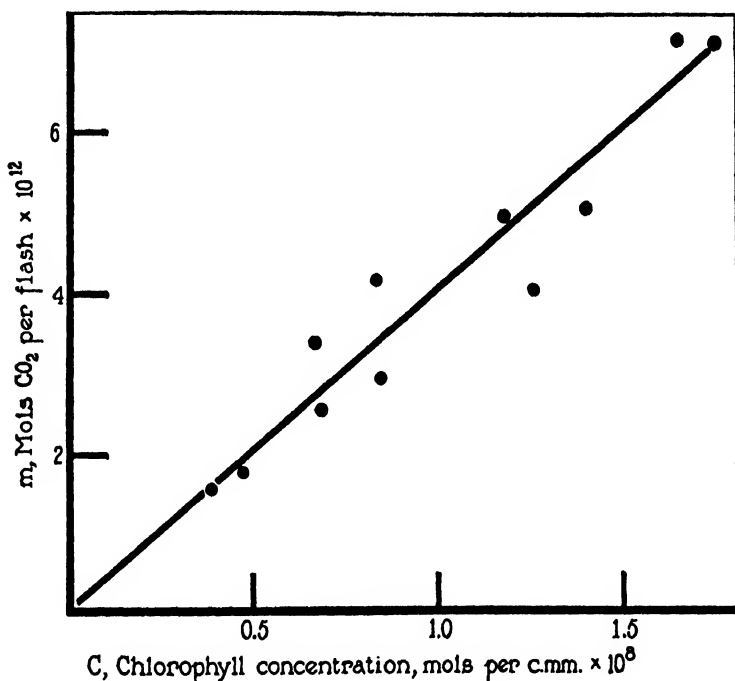


FIG. 2. Chlorophyll concentration in mols per c.mm. of cells plotted against mols of carbon dioxide reduced per flash of light at saturation. Temperature 25°C.

the points in Fig. 2. The slope of the curve,  $\rho$ , is therefore a constant for different concentrations of chlorophyll. The value of  $\rho$  obtained by averaging the last column in Table II, 2480 molecules of chlorophyll per molecule of carbon dioxide reduced per flash, agrees exactly with the slope of the line chosen as the best fit for the points in Fig. 2.

Column 8 in Table II gives the values of  $Q$ , photosynthesis in continuous light. It is to be understood that  $Q$  does not represent light saturation in all cases, since we were not able to obtain light of suffi-

cient intensity to saturate certain samples of cells. Nevertheless, it is interesting to compare the highest obtainable values of  $Q$  with those for maximum photosynthesis per flash.  $Q$  is plotted against chlorophyll in Fig. 3. The points are more scattered than in Fig. 2. We attribute the scatter to varying capacities to carry on the Blackman reaction among different samples of cells, a factor which would not

TABLE I

*Photosynthesis in Flashing Light as a Function of Light Intensity. Data for Fig. 1*

Capacity of condenser  $\frac{1}{2}$   $\mu$ fd.

Resistance of charging circuit 3340 ohms.

Twenty-one flashes per sec.

3100 volts at rectifier.

Temperature 24.9°C.

Relative light intensity	$\Delta h$ , per 5 min. per c.mm. cells, corrected for respiration	$KO_2$	Oxygen per 5 min. per c.mm. cells	Relative rate of oxygen production, $M$
First experiment				
	mm.		c.mm.	
100	1.16	0.52	0.603	14.50
75	1.18	0.52	0.614	14.75
50	0.99	0.52	0.515	12.40
28	0.68	0.52	0.354	8.50
10	0.25	0.52	0.130	3.15
Second experiment				
10	0.27	0.50	0.135	2.79
28	0.84	0.50	0.420	8.60
50	1.10	0.50	0.550	11.20
75	1.35	0.50	0.675	13.85
100	1.35	0.50	0.675	13.85

influence saturation in flashing light because the dark periods were sufficient for the completion of the Blackman reaction between flashes, but which would surely affect the balance between photochemical and Blackman reaction in continuous light. This might also explain why we could not saturate certain samples with continuous light. The cells with low chlorophyll content tended to fall short of saturation, even in our most intense continuous light. If cells with high

chlorophyll content should have their capacity for the Blackman reaction less well developed in proportion to their chlorophyll content than the cells with low chlorophyll content, then the cells rich in

TABLE II

*Photosynthesis in Flashing and Continuous Light as a Function of Chlorophyll Content. Data for Figs. 2 and 3*

Capacity of condenser  $\frac{1}{2}$  or 1  $\mu$ fd.

Resistance of charging circuit 3300 to 7500 ohms.

Twelve flashes per sec.

3100 volts at rectifier.

Temperature 25°C.

$\epsilon$ for 10 c.mm. cells, 25 cc. methanol	Source of light for culture	Chlorophyll per c.mm. cells $\times 10^3$	C Mols of chlorophyll per c.mm. cells $\times 10^3$	$KO_2$		Continuous light		Flashing light		$\rho, \frac{C}{m}$
				Continuous	Flashing	$\Delta h$ , per 5 min. per c.mm. cells corrected for respiration	$Q$ Mols oxygen per sec. per c.mm. cells $\times 10^{10}$	$\Delta h$ , per 5 min. per c.mm. cells corrected for respiration	Mols oxygen per flash per c.mm. cells $\times 10^{13}$	
		mg.				mm.		mm.		
0.0816	Neon	0.428	0.472	0.41	0.50	3.26	1.99	0.286	1.77	2660
0.204	Mercury	1.07	1.18	0.41	0.52	5.78	3.53	0.768	4.95	2380
0.146	Neon	0.766	0.845	0.41	0.50	6.05	3.69	0.473	2.93	2380
0.242	Mercury	1.27	1.40	0.41	0.52	6.87	4.18	0.780	5.03	2780
0.0672	Neon	0.352	0.389	0.41	0.41	1.28	0.72	0.308	1.57	2480
0.144	Mercury	0.756	0.835	0.41	0.41	5.24	3.19	0.815	4.15	2010
0.0544	Neon	0.289	0.314	0.41		2.20	1.34			
0.250	40 watt lamps	1.31	1.45	0.41		6.06	3.70			
0.0423	Neon	0.222	0.245	0.41		2.73	1.67			
0.224	40 watt lamps	1.17	1.29	0.41		5.15	3.14			
0.118	Neon	0.620	0.672	0.52	0.52	2.70	2.08	0.392	2.53	2660
0.218	Mercury	1.14	1.26	0.50	0.50	4.57	3.40	0.650	4.03	3120
0.116	Neon	0.606	0.668	0.52	0.52	5.03	3.89	0.525	3.38	1980
0.284	Mercury	1.49	1.65	0.50	0.50	6.16	4.58	1.152	7.15	2310
0.303	Mercury	1.59	1.75	0.50	0.50	6.05	4.50	1.142	7.10	2460
								Average $\rho \dots$		2480

chlorophyll would be saturated with light at lower intensities than those poor in chlorophyll. This would explain the direction of curvature of the line drawn through the points in Fig. 3. It would also explain the shape of the chlorophyll-photosynthesis curves published by Emerson (1929) for *C. vulgaris*. Those curves were made with cul-

tures of the same age and nearly the same density. The maintenance of equal age and density in cultures of *C. pyrenoidosa* grown over mercury and neon lights is not as easily possible, since the cultures mature so much faster in the neon light. The capacity for the Blackman reaction may well depend on the age and rate of growth of the culture, as well as on the chlorophyll content.

We are aware that we have published curves (1932, p. 413, Fig. 12) indicating identical capacity for the Blackman reaction relative to

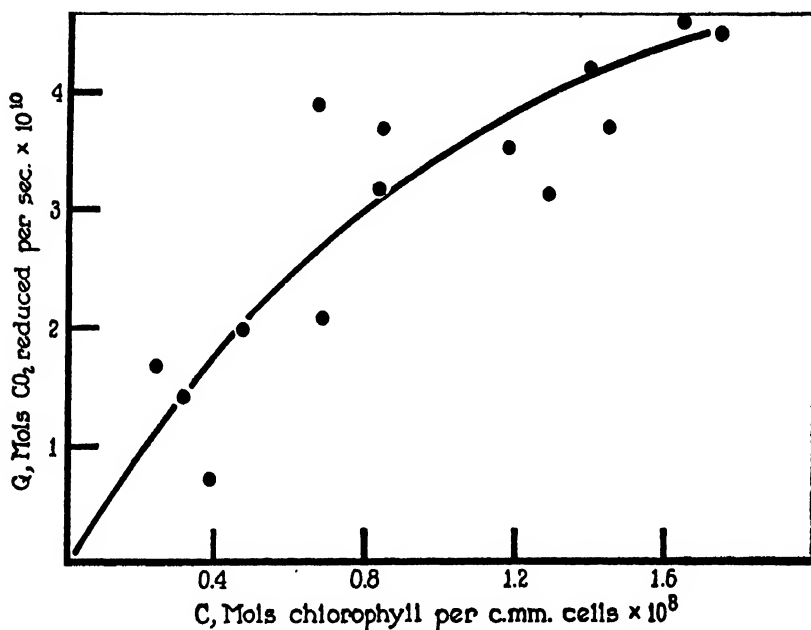


FIG. 3. Concentration of chlorophyll in mols per c.mm. of cells plotted against mols of carbon dioxide reduced per second in high intensity continuous light. Temperature 25°C.

chlorophyll content in two very different samples of cells, grown over red and blue light. We feel that we must attribute this result to chance, because the factors governing the development of the capacity for the Blackman reaction are evidently not yet under our control.

#### IV

##### *Theoretical*

We can give no adequate interpretation of our ratio of 2480 molecules of chlorophyll per molecule of carbon dioxide reduced per flash.

Warburg and Negelein (1923) found that under favorable conditions *Chlorella pyrenoidosa* could reduce one molecule of carbon dioxide for every four quanta absorbed. The light emitted by our neon tube is rich in red, a color strongly absorbed by chlorophyll; and we know that at high intensities the yield of photosynthesis per unit time of light is greatly improved by illuminating with short flashes separated by long dark periods. As yet we know nothing of the quantum efficiency in flashing light. We are forced to conclude that this is very low, or that most of the chlorophyll is not absorbing light. The fact that our maximum intensities nearly saturated the photochemical reaction does not mean necessarily that every chlorophyll molecule was absorbing light in each flash. We need only suppose that for every 2480 molecules of chlorophyll there is present in the cell one unit capable of reducing one molecule of carbon dioxide each time it is suitably activated by light. At our maximum intensity each flash activated nearly all these units.

It is also possible that the molecular weight of chlorophyll in the cell may be higher than that of extracted chlorophyll.

Knowing the number of units and the maximum rates of photosynthesis in continuous and flashing light, we can calculate the average time required for one unit to go through the cycle of photochemical and Blackman reactions. We suppose the Blackman reaction must be completed each time the photochemical reaction takes place, before the unit involved is again free to undergo the photochemical reaction. Therefore the mean time of one cycle, which we call  $S$ , will be longer at low temperatures than at high ones.

To calculate  $S$ , we shall let  $C$  be the chlorophyll content of the cells;  $m$  the photosynthesis per flash at saturation; and  $Q$  the photosynthesis per second at saturation with continuous light.  $C$ ,  $m$ , and  $Q$  are in mols per c. mm. of cells. We have used  $m$  as a measure of the number of units capable of undergoing the photochemical reaction. If  $S$  is the mean time required for a unit to undergo photochemical and Blackman reactions, then  $m/S$  is the maximum possible rate of photosynthesis in continuous light at saturation, so we may write

$$Q = \frac{m}{S}, \text{ or } S = \frac{m}{Q}.$$

For a sample of cells whose values of  $m$  and  $Q$  fall on Curves 2 and

3,  $m$ , which is independent of temperature, is  $2.52 \times 10^{-12}$  mols. At  $25^\circ$   $Q$  is  $2.08 \times 10^{-10}$  mols. From these figures  $S$  is equal to  $1.2 \times 10^{-2}$  sec.

We can also estimate the value of  $S$  from curves showing the duration of the dark reaction after a flash of light. The mean time of one cycle (neglecting the duration of the light reaction because it is very short compared to that of the dark reaction) will be approximately equal to the time required for the Blackman reaction, when taken by itself, to convert half the product of the photochemical reaction. This time can be read from numerous curves for the dark reaction which we have published previously (1932) for temperatures below  $25^\circ$ . Fig. 8 (1932, p. 403) shows that at  $25^\circ$  the Blackman reaction is substantially completed in less than 0.035 sec., the shortest dark time used. It would be half completed in about half this time, the exact point being determined by the order of the Blackman reaction, which we do not yet know. But we may say from Fig. 8 that  $S$  is about 0.017 sec., a figure of the same order of magnitude as the value calculated from  $\frac{m}{Q}$  at  $25^\circ$ . The value of  $Q$  for lower temperatures can be calculated roughly from the known temperature coefficient of photosynthesis, and the resulting values of  $S$  remain of the same order of magnitude as those estimated from the dark-time curves for corresponding temperatures.

We conclude, then, that at  $25^\circ$  the mean time required for a unit to complete the cycle of photochemical and Blackman reactions, reducing one molecule of carbon dioxide, is somewhere between 0.01 and 0.02 sec.

Now we shall present evidence, derived from the preceding experiments, that the photochemical reaction is of the first order with respect to light intensity. We will designate the number of units ready to undergo the photochemical reaction, as they would be after a long dark period, by  $N$ . Under the influence of light some of these units are activated and become ready to undergo the Blackman reaction. We will designate the number of these photoactivated units as  $n$ . They are reconverted by the Blackman reaction. We will let  $K$  be the value of  $N + n$ .  $K$  is a constant for a given sample of cells, and is proportional to the chlorophyll content of the sample. We assume

also that the rate,  $R$ , at which the units undergo the photochemical reaction, is proportional to the light intensity and to the value of  $N$ . We need not make any assumptions about the reconversion of the units as long as we consider photosynthesis in flashing light with long dark periods.

Our assumptions are:

$$N + n = K, \quad (1)$$

and

$$R = AIN. \quad (2)$$

In equation (2)  $A$  is the velocity constant for the reaction  $N + h\nu \rightarrow n$ . Equation (2) can be expressed in differential form:

$$\frac{dN}{dt} = - AIN, \quad (3)$$

with  $t$  representing the time, neglecting the Blackman reaction because  $t$  is short.

Integrating both sides of (3),

$$N = ce^{-\int AIdt}. \quad (4)$$

When  $t = 0$ , equation (4) becomes

$$N = c,$$

but we know that after a long dark period, all the units are ready to undergo the light reaction, so  $n = 0$ , and  $N = K$ . Hence we may replace the integration constant  $c$  in equation (4) by  $K$ .  $A$  is a constant, and may be written before the integral sign:

$$N = K e^{-A \int Idt}. \quad (5)$$

We have good evidence that  $E$ , the total energy in a flash, is equivalent to  $\int I dt$ . By inserting different sized choke coils in series with the condenser and the neon tube, the light flashes can be made slower and less intense, while their energy content,  $E$ , remains proportional to the charge on the condenser, a constant. By means of our spinning thread (Emerson and Arnold, 1932, p. 397) we estimated that our



largest choke coil increased the duration of the flash about 100 times. The maximum intensity of the flash is correspondingly decreased since the total energy liberated is the same. Photosynthesis was measured in flashes of various duration while  $E$  was kept constant. Unfortunately the fact that the energy emitted by the tube remained constant does not mean that the energy absorbed by the cells was the same for the various flashes. The wave length distribution of the flashes differed with the different choke coils. The slower flashes were darker red, and may have emitted more energy as heat. For this reason the results are not quantitative. Nevertheless, we were only able to find differences of 9 per cent in the photosynthesis per flash, using the largest choke coil. These experiments indicate that it is correct to use the first power of the intensity in equation (5). A higher power of  $I$  would require that the amount of photosynthesis per flash change with changes in intensity and duration of flash produced by the different choke coils. It appears from the experiments that  $\int I dt$ , which remains equal to the charge on the condenser, a constant, produces the same photosynthesis even when the time and intensity are changed over a wide range.

We will now let  $N_1$  denote the value of  $N$  remaining at the end of a light flash, and rewrite equation (5):

$$N_1 = K e^{-A \int I dt}. \quad (6)$$

$K - N_1$  will be the number of units activated, measured by  $M$ .

$$M = K - N_1,$$

or

$$M = K - K e^{-A \int I dt}. \quad (7)$$

Subtracting both sides of equation (7) from  $K$ , and simplifying,

$$\frac{K - M}{K} = e^{-A \int I dt}.$$

or

$$\log \frac{K - M}{K} = -A \int I dt. \quad (8)$$

According to equation (8), the log of  $\frac{K - M}{K}$  plotted against  $\int I dt$  should give a straight line of slope  $-A$ , intersecting the logarithmic axis where  $M = 0$ . This plot can be made from Fig. 1.  $\int I dt$  is proportional to the intensity scale, and  $M$  is the height of the curve at any point.  $K$ , being proportional to  $C$ , the chlorophyll content, is proportional to the maximum height to which the curve rises. It was mentioned in the discussion of Fig. 1 that our light intensities did not

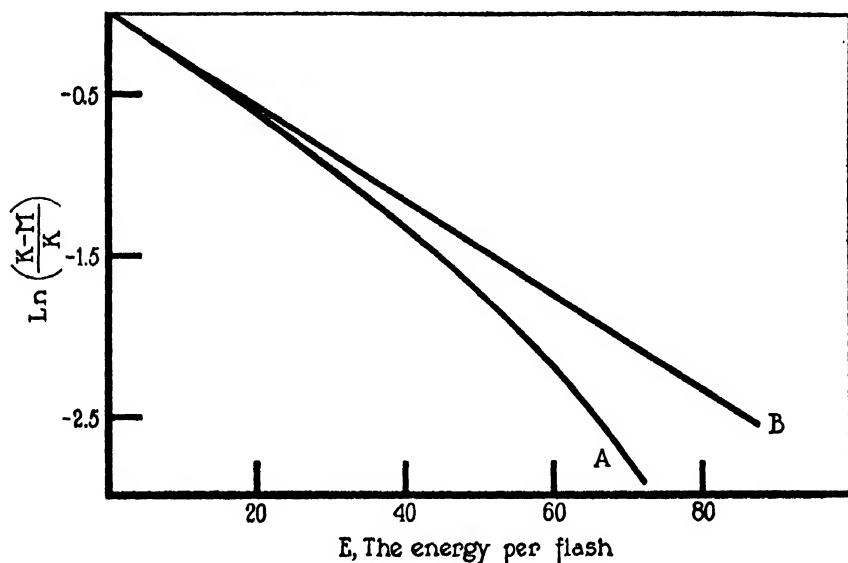


FIG. 4. See text for explanation. Curve A is made from Fig. 1, taking  $K$  as the maximum height, 14.5, attained by the curve drawn in Fig. 1. Line B is plotted from Fig. 1 on the assumption that  $K$  would finally reach the value 15.5, if we could obtain flashes of sufficient intensity.

permit the experimental determination of this maximum height. We may assign to  $K$  either the highest value attained by Curve 1, or a slightly higher value, the probable maximum. Curve A in Fig. 4 is a plot of  $I$  against  $\log \frac{(K - M)}{K}$  when  $K$  is given the value 14.5, the highest level actually attained by Curve 1. Curve A deviates most from a straight line in the region where too low a value of  $K$  would make the largest error. If we assign to  $K$  the value 15.5 instead of 14.5, we obtain the line B, in Fig. 4, as required by equation (8). Since 15.5 is probably nearer the maximum height attainable by the

curve in Fig. 1 than 14.5, we think our interpretation comes close to fitting the experimental results.

Any interpretation in which light intensity enters this mechanism at a power higher than the first, fails to explain these results as satisfactorily. If we retain the concept that there are units in the photosynthetic mechanism which go through the photochemical and Blackman reactions in a cycle, our explanation is a good fit. Future work may show that we are mistaken in interpreting the behavior of photosynthesis in flashing light to mean that a cyclical type of reaction is governing the process. In this case our scheme will have to be abandoned. It covers the photochemical reaction only, but gives promise that it may be extended to include the Blackman reaction as well. Qualitatively it will explain the shape of the ordinary intensity curves published by Warburg (1925), van den Honert (1930), van der Paauw (1932), and others. We suppose that saturation in continuous light is reached when the photochemical reaction produces its product as fast as the Blackman reaction can use it. But an exact interpretation of the balance between the photochemical and Blackman reactions must await a better understanding of the Blackman reaction.

#### SUMMARY

Measurements of photosynthesis were made in continuous and flashing light of high intensity, using cells varying in chlorophyll content. The amount of chlorophyll present per molecule of carbon dioxide reduced per single flash of light was found to be about 2480 molecules. The length of time required for one unit in the photosynthetic mechanism to complete the cycle of photochemical and Blackman reactions was found to be about 0.02 sec. at 25°C. The equation  $R = AIN$  was shown to give a good description of the rate of the photochemical reaction, when  $A$  is a velocity constant,  $I$  the intensity of light, and  $N$  the number of units in the photosynthetic mechanism.

We are greatly indebted to Mr. Erickson and to the Electrical Products Corporation for the large number of tubes which they furnished us. Our thanks are due especially to Professor R. C. Tolman for helpful criticism.

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# THE EFFECTS OF RADIATIONS ON BIOLOGICAL SYSTEMS

## I. INFLUENCE OF HIGH-FREQUENCY X-RAY RADIATION UPON THE DURATION OF THE PREPUPAL PERIOD OF *DROSOPHILAE*

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Some results of investigations conducted in this laboratory concerned with the influence of x-ray irradiation upon the duration of the prepupal period of *Drosophila melanogaster* have been reported<sup>1,2</sup> previously. In every instance an extension of the larval period resulted. In the case of high-frequency radiation, investigations of the course of such effects could not be extended as much as was desired, on account of the low power of sources then available. However, over the early part of the course it was definitely indicated<sup>1</sup> that the duration of the prepupal period was an increasing function of the time of irradiation under the given conditions. In the absence of more powerful sources investigations of effects of more extensive irradiation were conducted<sup>2</sup> with the inclusion of radiations of lower frequency in order to increase the available power. The demonstration of a maximum point of effectiveness of prolonged irradiation (in extension of the mean prepupal period,  $\phi$ ) was a striking result. It is the purpose of the present communication to give the results of similar experiments employing high-frequency radiation from a more powerful source.<sup>3</sup>

### *Preparation and Maintenance of Larvae*

The *Drosophilae* employed in the present and former work<sup>1,2</sup> were raised in distinct generations from an original culture obtained from Dr. J. H. Northrop

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<sup>1</sup> Hussey, R., Thompson, W. R., and Calhoun, E. T., *Science*, 1927, **66**, 65.

<sup>2</sup> Tennant, R., *Science*, 1931, **73**, 567.

<sup>3</sup> A Coolidge water-cooled tube kindly supplied by the General Electric Co. (General Electric X-Ray Corp.).

who had grown the strain for many generations under aseptic conditions, and have been maintained under similar conditions at 25°C. (approximately within 1°) in this laboratory. The stock food consisted of a mixture of 1 part of thoroughly ripened banana to 3 parts of yeast<sup>4</sup> by weight to which was added 2 ml. of glacial acetic acid for each 100 gm. of yeast used, the whole being autoclaved for 45 minutes on each of 2 successive days at approximately 121°C. This autoclaving technique was employed in preparing all foods, water, flasks or voile filters for use in the present work. Flasks for seeding and generation were prepared with side tubes for delivery of imagoes *via* an aseptic rubber tube ordinarily fitted with a glass plug; those for stock containing a sufficient amount of the food described above for maintenance upon absorbent cotton, and those for preparation of larvae for experiment containing 6 ml. of a food consisting of 15 gm. of yeast to 20 ml. of beef infusion agar (pH 7.0). The flasks were ordinarily Pyrex Erlenmeyer flasks of 125 to 200 ml. capacity; the neck being fitted with a plug of non-absorbent cotton wrapped in cotton gauze at its base and covered with a paper cap. Seeding flies (of age not exceeding 9.5 days) were used in every instance for an interval between 23.5 and 24.5 hours in a given flask.

In the case of stock flasks additional food was added 24 hours after removal of the parent flies. This food consisted of a mixture of 454 gm. of yeast and 400 ml. of distilled water plus 1 part to 50 by volume of glacial acetic acid, autoclaved in the usual way. 10 ml. of this food were used (added as stated above through the delivery tube) for each stock flask, and in the case of the yeast-agar flasks (for experimental larvae) 4 ml. of this food were added to each flask immediately after removal of the parent flies, and the flask left tilted at an angle of about 60° from the normal vertical position for 24 hours, after which it was returned to the vertical position.

Ordinarily, 4 days after the commencement of generation the larvae of the yeast-agar flasks were ready for experimentation. The median age of larvae at any given instant is taken as the time elapsed since the mid-point of the seeding interval,  $\alpha$ . Thus, ordinarily, irradiations were started at a time when  $\alpha = 3.5$  days, approximately, which was the same in earlier work so far reported.<sup>1,2</sup> The larvae were washed with sterile tap water onto sterile voile filters and freed from food and extraneous matter with further washings and the aid of camel's hair brushes. Then the larvae were distributed in random samples in wells in blocks made of stearin-paraffin (1 part by weight of stearic acid to 3 parts of paraffin—the melting point of the latter being 52–54°C.). The wells were cut in a plane surface, and were cylindrical in shape (diameter = 25 mm. and 5 mm. deep). They had three pieces of filter paper (Whatman No. 2) fitted in the bottom upon which the larvae were placed. Then 0.1 ml. of sterile tap water was added to each well and a cover of filter paper permeated with stearin-paraffin sealed over its top. Unless otherwise specified, the cover was then perforated with a needle in a uniform manner so as to permit ventilation. Aseptic technique was employed throughout.

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<sup>4</sup> The yeast was supplied by the Fleischmann Yeast Co.

All stearin-paraffin blocks were of standardized thickness, 25 mm., and those used in irradiations were arranged so that alone or fitted together as the case might be they formed a rectangular prism  $250 \times 250 \times 25$  mm. with the plane surface (250 mm. square) uppermost in horizontal position. In the case of all but the depth experiments the wells were cut in this surface. In the depth experiment a slight difference existed in order to permit ventilation even when such blocks were stacked as will be described later.

In all irradiations a stack of blocks of this type was set so as to be inscribed in a rectangular prism with horizontal cross-section 250 mm. square and what will be called its *axis of reference*, through the centers of these cross sectional squares, vertical and passing through the center of the target of the x-ray tube. In describing the situation of wells the distance between the axis of a given well and this axis of reference when in position for irradiation will be called  $d$ , and the vertical distance (difference in elevation) between the target center and well top will be called  $D$ . The axis of any well in arrangements for irradiation was always in a plane through the ideal circumscribing prism's diagonally opposite vertical edges, and the prism arranged so that such a plane made an angle  $\theta$ , with the axis of the x-ray tube (which was always in the horizontal position customary for direction of radiations for use vertically beneath the tube),  $\theta$  being  $45^\circ$  in the case that will be called *Phantom Form 1* and being equal to either  $0$  or  $90^\circ$  in *Phantom Form 2*. Furthermore, in order to avoid circumlocution in describing irradiations, in addition to the above definitions let  $Z$  be the distance of a given point below the uppermost horizontal plane of the above mentioned circumscribing prism; and in particular, let the value of  $Z$  for the points on the corresponding lowest plane be called the *phantom depth*, and the value of  $Z$  for the uppermost points of a given well be called its *depth in the phantom*,  $\lambda$ . Thus for wells situated at the upper plane surface of the phantom,  $\lambda = 0$ , which was the case in most of the experiments to be described below.

The details of irradiation technique will be given separately in description of particular experiments using the present outline as a foundation in order to save repetition where treatment was uniform as was the case following irradiations in that control or irradiated larvae were transferred to small wide-mouthed flasks (as nearly at the same time as practicable) containing enough yeast food on cotton for their maintenance while under observation, and subsequently observed at definite intervals (about  $\frac{1}{2}$  day) for pupation, all pupae formed being removed, counted, and the number for each class recorded together with the time of observation. Thus, in an obvious manner, the mean age of pupation,  $\phi$  (estimated from the mean time of seeding as is  $\alpha$ ) or the corresponding median time of pupation,  $\phi'$ , could be calculated.

As in preceding work, the greatest care practicable was exercised to prevent contamination. In the experiments reported, transfers were made of flask contents at the end of pupation observations to beef infusion broth and kept at  $37.5^\circ\text{C}$ . for 48 hours, during which time they were observed at intervals without ever detecting growth.



Instead of continuing observation until all larvae had either pupated or died, the alternative of cessation in accord with an arbitrary criterion was adopted; namely, if an interval of more than 1.9 days elapse in which it be observed that less pupae were formed than one-twentieth of the total formed in a given lot, then further observations on this lot were excluded in calculation. This instituted much saving in time and space required for the work without altering the averages greatly (mean or median) and affected increments in such averages as the result of irradiation or other treatment even less.

#### EXPERIMENTAL

Experiments were carried out in a manner similar to those reported<sup>1,2</sup> previously to trace the course of variation of the median prepupal period,  $\phi'$ , with duration of irradiation,  $t$ , under otherwise fixed conditions. In terms of the variables defined above these were as follows:

Radiations generated by a Coolidge water-cooled tube impressed by 191 kv. (peak) with current of 30 ma., were passed through a horizontal circular aperture 380 mm. below the target center and 180 mm. in diameter and through filters immediately beneath this of 0.5 mm. of copper and 1.0 mm. of aluminum to the phantom in Form 2 (phantom depth = 175 mm.,  $\lambda = 0$ ,  $D = 500$  mm., and  $d = 60$  mm.). As in all previous work the top layer of the phantom consisted at all times (in course curve investigation) of four blocks, 25 by 125<sup>2</sup> in millimeters, with well of standard type drilled in the upper square surface 60 mm. along a diagonal from a corner placed in touch with the similar corners of the companion blocks. Eight such blocks were used in each experiment; No. 8 being the unirradiated control, No. 7 being irradiated for the full irradiation time of all, and the pairs, 1 and 6, 2 and 5, and 3 and 4 having the sum of irradiation intervals for each pair equal to that of No. 7. Thus all irradiations in a given experiment were performed during the irradiation of No. 7 by successive replacements with slight interruption; the chronological order of the replacements being reversed in alternate experiments of this sort and the orientation of the pairs in irradiation being varied so as to minimize effect upon the means of several experiments which might be involved. The results of four experiments of this type are given in Table I and represented graphically in Text-fig. 1, where the presence of a maximum point is obvious as is the subsequent decline in effective-

ness from the point of view of increasing the larval period to an almost level plateau, the curve being much like that already presented<sup>2</sup> in the case of unfiltered radiation. The mean and median prepupal periods give essentially the same form of curve (both are given in the case of the first experiment of Table I), but the median alone will be used in work described below because of greater simplicity in the making of necessary observations and calculations.

In connection with these course curves (Text-fig. 1) it should be stated that in the first report<sup>1</sup> on such work any attempt to approximate the mean prepupal period,  $\phi$ , by an asymptotic logarithmic curve

TABLE I

*Influence of the Period of Irradiation upon the Duration of the Prepupal Period*

Experiment..... 1		1	2		3	4
$t$	Mean $\phi$	Median $\phi$	Median $\phi$	$t$	Median $\phi$	Median $\phi$
0	(5.82)	5.73	5.65	0	5.16	5.21
40	(7.13)	6.90	7.10	50	7.33	7.27
80	(8.96)	8.84	9.19	65	8.18	8.40
120	(8.49)	8.41	8.69	80	8.76	9.00
160	(7.84)	7.72	7.82	95	9.62	9.36
200	(7.66)	7.61	7.70	110	9.69	9.41
240	(7.75)	7.54	7.71	125	9.32	8.77
280	(7.77)	7.69	7.72	175	8.27	7.96

as an increasing function of the period of irradiation,  $t$ , was definitely avoided; as was any attempt to explain the results upon the basis of destruction of enzymes in irradiation, which subject has been studied, however, as another means of approach to an understanding of the influence of radiations upon biological systems.<sup>5</sup> Indeed, evidence already at hand but which was considered unready for publication at that time led to the work previously mentioned<sup>2</sup> in which more extensive x-ray irradiations were obtained by eliminating the copper and aluminum filters used in the present work. The form of the curves thus obtained,<sup>2</sup> together with those of the present experiments (Text-fig. 1) definitely contradict the proposition of fit of  $\phi$  to an increasing

<sup>5</sup> Hussey, R., and Thompson, W. R., *J. Gen. Physiol.*, 1922-23, 5, 647; 1923-24, 6, 1, 7; 1925-26, 9, 211, 217, 309, 315; 1931-32, 15, 9.

exponential function of  $t$ . However, another author<sup>6</sup> has employed the data of our earlier work<sup>1</sup> for this purpose and in his presentation

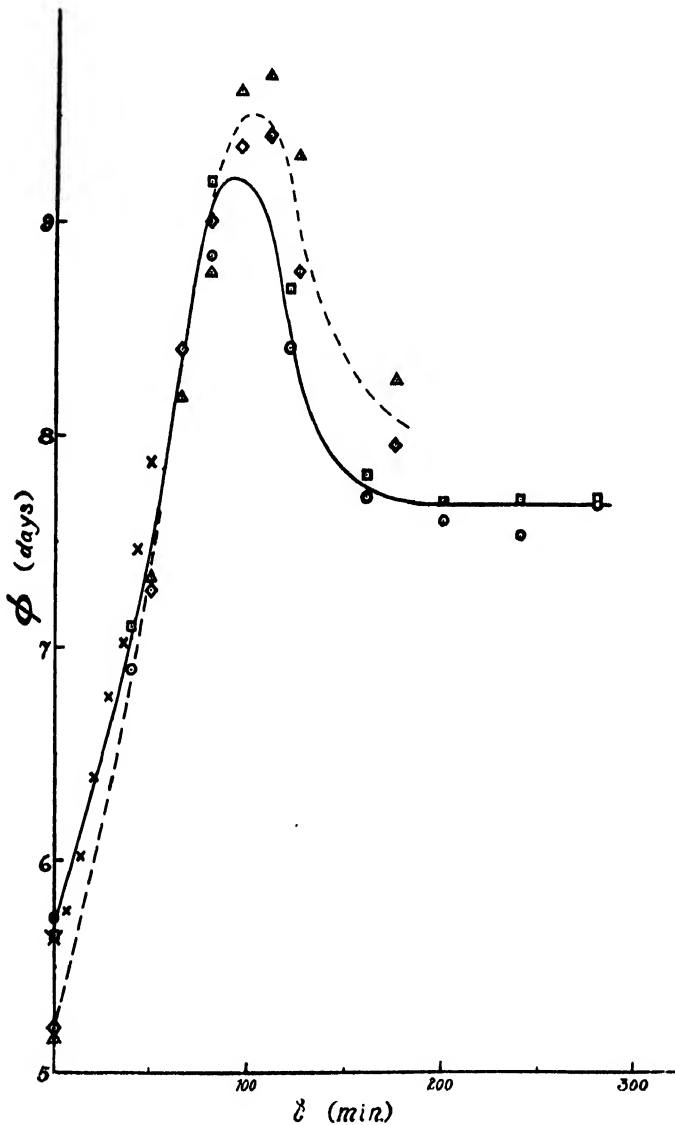


FIG. 1

the approximation appears fairly close. It happens only too often in presentation of biological data that attempts are made to represent

<sup>6</sup> Koidsumi, K., *J. Soc. Trop. Agric.*, 1930, 2, 258.

the observations by simple logarithmic curves, apparently as a result of the success with which they have been employed in physical chemistry. Brooks<sup>7</sup> and others have emphasized this fact. In some instances cited by Brooks even the exclusion of the early part of a series of observations as a *period of incubation* has been undertaken in order to fit the data to the favored curve, but in the present instance the difficulty presents itself in the form of a *maximum point* in the middle of the experience.

In Text-fig. 1 the data of the former experience<sup>1</sup> (with approximately the same quality of radiation as at present employed) in the study of the course of effect of irradiation upon the prepupal period are given with the abscissae equal to the time of irradiation times the factor,  $\frac{5 (50)^2}{30 (54)^2}$ , which is the estimated relative intensity of radiation in the former work. The points are indicated by the cross (X) in the figure, where the approximation to the present results on this basis in spite of technical differences is obvious particularly as to the slopes of the curves. This indicates the possibility that within wide limits a close approximation of the same results may be had when the intensity and time of irradiation are varied inversely under otherwise fixed conditions as to quality of radiations and technical handling. Experiments dealing with such relations have been planned for future work.

Another series of experiments were carried out with wells in a phantom of the same dimensions as that for the course curve but with a well in the center ( $d = 0$ ) of the square upper face as well as the four at  $d = 60$  mm. and with the phantom in the *Form 1*. The well-block was made as a single piece ( $25 \times 250^2$  mm.) containing the five wells as all irradiations were planned to be equal and simultaneous. These wells and the larvae lots irradiated in them will be designated as follows:

A—coaxial with the phantom and radiation cone, B and D having their line of centers parallel with the axis of the x-ray tube, B on the cathodal side (North), and D on the anodal side (South) relative to A, and C and E being in the relative *West* and *East* positions, respectively. Thus the four wells lay (relative to centers) on a circle of radius equal

<sup>7</sup> Brooks, S. C., *J. Gen. Physiol.*, 1918-19, 1, 61.

to 60 mm. in quadrant positions and with A at the center vertically beneath the target centroid. Aside from the period of irradiation which was 60 minutes in each instance, other conditions were as described for the course curve experiments above, including a control, S. For the sake of brevity, the values of  $\phi$ , the median prepupal period, observed in four such experiments are not given in Table II but rather the corresponding values of the ratio ( $R$ ) of the excess of these values over those for S to the mean of such excess for the four symmetrically placed wells (B, C, D, and E) in each experiment, which furnishes a more satisfactory basis of comparison. Indeed, in view of the results

TABLE II

*Relative Effectiveness of Radiations at Given Points on the Upper Surface of the Phantom*

Position	A (central)	60 mm. from the center			
		B (cathodal) north	C (medial) west	D (anodal) south	E (medial) east
Experiment	$R$	$R$	$R$	$R$	$R$
1	1.16	0.98	1.04	1.01	0.98
2	1.09	1.04	0.97	0.96	1.03
3	1.06	0.98	1.03	0.92	1.07
4	1.09	0.98	1.00	0.98	1.05
Mean R. ....	1.10	1.00	1.01	0.97	1.03
A. D. ....	0.015	0.013	0.013	0.014	0.014

given for the course curve (in this region being approximately linear)  $R$  may be taken as an indication of relative effectiveness. No significant difference appears in the case of the four symmetrical wells, but about 10 per cent greater effectiveness at the center position, A, is indicated with an A. D. of approximately 1.5 per cent in each instance.

An unusually interesting result was observed in a companion to the above experiments, the same arrangement being used except that Well A was left empty and C and B were not provided with ventilation as in the case of an additional control. The period of simultaneous irradiation was 50 minutes under conditions otherwise essentially the same as in the above mentioned experiments of Table II, with the following results:

	Control ( $\phi_o$ )	Irradiated ( $\phi$ )	Mean ( $\phi-\phi_o$ )
Ventilated .....	5.81	7.74, 7.75	1.94
Unventilated .....	5.92	6.87, 6.86	0.95

The mean number of pupae removed per well was about 450 in the above experience, being 528 for the regularly ventilated control and 395 for the other, and for B, C, D, and E being 428, 429, 472, and 458, respectively. The approximate halving of the effect by omission of ventilation during irradiation appears significant. A qualitative difference in the distribution of larvae in the wells when they were unsealed was so marked that it seems worthy of mention that independently of irradiation (control and irradiated lots alike) in the wells whose covers were unperforated the larvae were massed upon the bottom, almost all being in one contiguous heap, whereas in those wells having covers perforated in the usual manner, the larvae were greatly dispersed about the cover, sides, and bottom.

Another experiment was performed (in duplicate) employing the same conditions of irradiation as above, all well covers being perforated as will be implied unless otherwise stated hereafter. The larvae in the four wells simultaneously irradiated, however, differed in median age by successive intervals of 0.5 day, having parents from the same generation allowed to seed flasks at intervals in advance fixed so as to make this possible, a control well corresponding to each well irradiated being maintained. The results are given in Table III, where a correlation of increase in both  $\phi$  and  $\phi_o$  with age at the time of the confinement in the wells is apparent,  $\phi_o$  being the median prepupal period for the control and  $\phi$  that for the irradiated lot of larvae (here in one to one correspondence).

In the previous report<sup>1</sup> of effects of high-frequency x-ray radiation on  $\phi$  it was pointed out that the larval system in paraffin or other phantom wells might be used as a means of estimating the effectiveness of radiations from a given source at different depths in such phantoms. The need of uniform ventilation in such studies is obvious in view of the results of the ventilation experiment described above. If stacks of blocks (each  $25 \times 250^2$  mm.) are to be used as in the phantoms so far employed in this laboratory, a convenient form for the well-blocks (which may be used for either depth or upper surface irradiations) is the following:

The regular size ( $25 \times 250^2$  mm.) phantom unit block of stearin-paraffin mixture with a cylindrical well (3 mm. deep and 50 mm. in diameter) cut in the center of the upper square face with another well cut coaxially in this well of the usual type for holding the larvae to be irradiated (5 mm. deep and 25 mm. in diameter) and a rectangular groove cut in the same face (3 mm. deep and 10 mm. in width) with sides parallel to plane faces of the block.

Obviously, larvae may be sealed in the small well in the usual manner, the cover perforated, and the block placed in any desired position

TABLE III

Variations in effects of a uniform treatment of larvae in different stages of development, comparing correlated effects in control and irradiated lots.

Experiment	Median age at time of irradiation ( $\alpha$ )	Control ( $\phi_0$ )	Irradiated ( $\phi$ )	$(\phi - \phi_0)$
1	3.0	5.36	7.36	2.00
2		5.43	7.22	1.79
1	3.5	5.61	7.48	1.87
2		5.67	7.19	1.52
1	4.0	5.71	7.68	1.97
2		5.84	7.68	1.84
1	4.5	5.84	8.09	2.25
2		5.89	7.79	1.90

in the stack (*e.g.*, in the seven-block phantom) with the groove in a conventional position. In the experiments to be described this groove was always parallel to the x-ray tube axis, Phantom Form 2 being used. Thus ventilation could be provided by means of a fan, the groove and large well forming a ventilation system when a block was placed above it as all block bottoms are plane in the system described. This arrangement was more satisfactory than that used in the experiments reported previously<sup>1</sup> and has been employed uniformly in investigations of effectiveness of radiations at depths in this phantom.

After certain preliminary investigation it appeared as if the effectiveness of radiations of the quality and intensity used in the work

described above might be approximately 0.82 times as effective at a depth of 25 mm. as on the upper surface in the phantom (Form 2) 50 cm. from the source. Accordingly, data were collected in four experiments in which two lots of larvae were irradiated in the top block position for 40 and 60 minutes respectively as in the course curve work, but in the standard central-vent well as employed at other positions and intermediately a lot of larvae was irradiated in the position of second block (25 mm. lower) for 61 minutes, all larvae in a given experiment being random samples from the same supply of larvae prepared as in the course curve experiments, approximately 3.5 days old. All larvae

TABLE IV

*Relative Effectiveness of Radiation at Top and at Depth of 25 Mm. in the Phantom*

Lot.....	A	B	C	S		
$\lambda$ .....	3 mm.	28 mm.	3 mm.		R	Deviation
t.....	40	61	60	0		
Experiment	$\phi$	$\phi$	$\phi$	$\phi_0$		
1	7.46	8.04	8.14	(5.97)	0.936	+.066
2	6.85	7.92	8.32	(5.56)	0.875	+.005
3	7.07	7.38	8.07	(5.59)	0.757	-.113
4	6.82	7.87	8.16	(5.63)	0.913	+.043
Mean.....	7.05	7.80	8.17	(5.69)	0.870 (A. D. = .028)	

and a control were placed in and removed from the wells at as nearly the same time as practicable and pupation observed as in previous work. The lots irradiated were designated A, B, and C in the order of irradiation, B being at the depth 25 mm. below that of A and C. The value of  $\lambda$  is, of course, 3 mm. greater, due to the cutting of the groove and large well system, but the results are treated as measures of approximate relative effectiveness of radiations at the depth 3 mm. higher.

This relative effectiveness ( $R$ ) may be estimated by means of a linear interpolation approximation of the time that would be required at the surface for the effect observed in B,  $R$  being the ratio of this time to the time actually required in the case of B. The results are given in Table IV, the mean value of  $R$  being 0.870 (A. D. = .028).



The value obtained by similar interpolation using the mean of the four values for each type of irradiation is 0.875, but this method of estimation is not as generally applicable as the former which does not require uniformity in the irradiation scheme.

The results so far presented (in Tables II and IV) obviously represent only a small portion of the phantom explorable in this manner, but are given as an illustration of a method of studying relative effectiveness of radiations in different portions of a phantom employing a living system as indicator. The contrast of such results with those similarly obtained with gaseous ionization chambers should be interesting in view of the controversial character the subject has assumed.

#### DISCUSSION

It is important to note that the shift in the average prepupal periods with irradiation in the above experiments is not accountable on the basis of selective mortality alone; and, indeed, the rôle of selective mortality in such effects seems to be insignificant. This is clearly brought out in the diagram of the distribution of pupation observed in the first course curve experiment (Table I) which is presented in Text-fig. 2. In the form of class-frequency diagrams (the height of the black rectangles being proportional to the number of pupae formed in the given observational interval, taken as  $\frac{1}{2}$  day) the distribution of  $\phi$  is given with the median value indicated by a white vertical line in each instance; the base line of each diagram being placed at the ordinate height relative to the period of irradiation,  $t$ . The representation is convertible to the conventional form in a three dimensional Cartesian coordinate system by the simple device of rotating each frequency diagram (blackened region) about its base line through an angle of  $90^\circ$  in a given direction.

Approximately the same number of larvae (about 300) were taken for each well (as stated previously), hence the approximation of the blackened areas to equality indicates the lack of great difference in the fraction of larvae reaching the pupal stage in these experiments. Thus it is obvious that the diagram would discredit any attempt to account for the observed shifts in the position of the median  $\phi$  upon the basis of selective mortality alone. The total pupae formed for the different lots (in order of increasing  $t$ ) were 287, 308, 313, 335, 268,

272, 265, and 270, respectively, in this experience. The value of the pupation-rate ordinate is not given in the figure, but the *distance* corresponding to 40 minute difference in  $t$  was taken to represent a pupa-

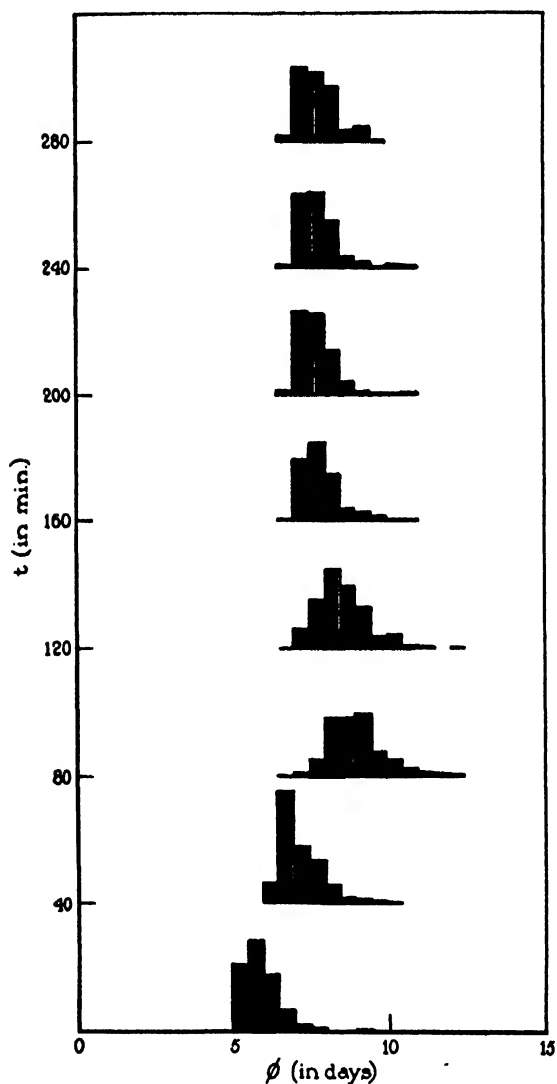


FIG. 2

tion rate of 150 per half day. This was done to avoid overlapping in the diagrams, the greatest rate observed being one of 133 pupae in a half day in the case of the lot irradiated for 40 minutes.

It is interesting to note the apparent decrease in skewness in the neighborhood of the maximum displacement of the average  $\phi$  (about an irradiation time of 80 minutes).

#### SUMMARY

The effect of high-frequency x-ray irradiation in prolongation of the larval stage of *Drosophila melanogaster* has been studied further, and evidence presented of the attainment of a maximum effect followed by a decrease to an almost level plateau in the course curve of average (median) prepupal period ( $\phi$ ) as a function of the period of irradiation ( $t$ ) under otherwise fixed conditions. The variation of effects of the experimental treatment with age of the larvae at the time of irradiation has been demonstrated in both control and irradiated lots, and a strikingly decreased effect observed when ventilation was not supplied as usual.

Means of employment of a living system of this type as an indicator of effectiveness of radiation as in phantom depth or other distributional experiments have been presented and their use illustrated.

# A STUDY OF THE EFFECTS OF CERTAIN VARIATIONS IN PREPARATION OF A STARCH SUBSTRATE IN AMYLASE VISCOSIMETRY

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In earlier studies in this laboratory on starch-amylase digestion, it was found that considerable variation existed in the physiochemical properties of starch prepared by the method of Litner. Accordingly, as a temporary expedient, experiments were restricted to a single batch of starch.<sup>1,2</sup> The following work was undertaken to ascertain the effect of dilution of the starch substrate, and to investigate the possibility of establishing relations between the various batches of commercial soluble starch, using relative viscosity and amylase digestivity as comparative factors.

In another communication<sup>1</sup> from this laboratory, a method has been described for the estimation of amylase by a viscosimetric method, employing 7 per cent starch solution (Baker's Soluble Starch, Lot No. 13128) as substrate, wherein the general aspects of viscosimetric methods have been discussed. In what follows it will be attempted to employ, wherever possible, the same symbols as were used in that discussion, for the variables involved. Briefly summarized these are as follows:

For a particular digestion,  $s$  is the time in seconds (to the nearest tenth) required for the digestion mixture in a given viscosimeter to flow between the fixed marks at a time,  $t$ , from the mid-time of this observation to the mid-time of mixing the enzyme and substrate.  $t$  is expressed in hours to the nearest thousandth.

$s_0$  is the mean time of outflow in seconds, previously estimated in the

<sup>1</sup> Thompson, W. R., Johnson, C. E., and Hussey, R., *J. Gen. Physiol.*, 1931-32, 15, 1.

<sup>2</sup> Johnson, C. E., and Wies, C. H., *J. Exp. Med.*, 1932, 55, 505

same viscosimeter for a solution differing from the digestion mixture only in that a conventional solution devoid of enzyme (*e.g.*, 0.85 per cent saline) is substituted for the enzyme solution in admixture with substrate.  $s_0$  is called, for brevity, the *zero outflow time* for the given digestion.

$\phi$  is defined as a positive number, less than unity and fixed for any enzyme substrate system, and  $S$  is defined by the relation

$$(1) \quad S = s_0 \phi$$

In the work<sup>1</sup> with the 7 per cent starch as substrate, the value  $\phi = 0.800$  was arbitrarily chosen. In these digestions as in those mentioned below, the temperature was maintained within 0.03 of a degree of 37.50°C. The associated values ( $t$ ,  $s$ ) for a given digestion form a set of points which may be plotted on coordinate paper and a smooth curve fitted as described in the report<sup>1</sup> previously mentioned. On this curve a point whose ordinate is  $S$  is found, and the corresponding abscissa is called  $T$ . It was found that  $T$  varied in inverse proportion to the enzyme concentration. Accordingly,  $Q$  was taken as the concentration of amylase (in the solution, 5 parts of which are mixed with 25 parts by volume of substrate) in arbitrary units, defined by the relation

$$(2) \quad Q = \frac{1}{T}$$

The actual concentration of amylase in the digestion mixture is estimated as 1/6 of this under the circumstances.

The object of the experiments of the first part of the present report was to show what value of  $\phi$  should be chosen for starch substrate solutions made from the same lot of starch as the above, but differing in the starch concentration, in order that  $T$  should be the same for these systems when the enzyme concentration is the same. Accordingly, simultaneous digestions were run with a standardized starch substrate in each instance, and another substrate solution with the same concentration of enzyme. From the data on the former digestion,  $T$  was estimated (as a mean of four digestion observations), and by an inverse process a point on the curves for the other substrate solution having the abscissa  $T$  was found. The corresponding ordi-

nate was called  $S$ , and the ratio of this to  $s_0$  (the corresponding *zero outflow time*) taken as an estimate of the appropriate value of  $\phi$  mentioned above; or, as an alternative which was employed after the first such experiment in which the standard 7 per cent substrate was compared with the 3 per cent starch substrate, for which the value  $\theta = 0.842$  was found appropriate, this 3 per cent starch was used as the basis of comparison of others. The mean of four such estimations is given in the instance of each substrate solution so employed, together

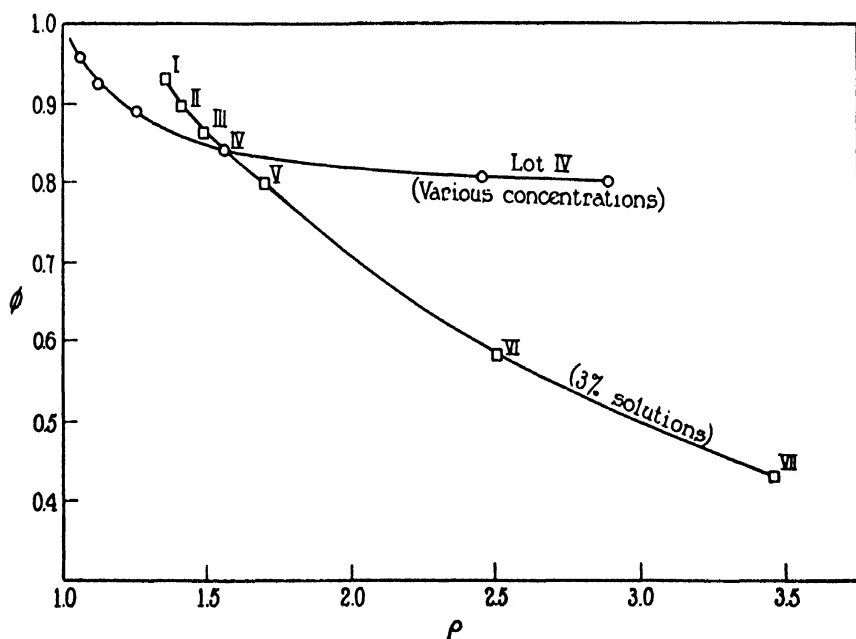


FIG. 1

with the corresponding mean value of  $\rho$ , the relative viscosity, defined by the relation

$$(3) \quad \rho = \frac{s_0}{W}$$

where  $W$  is the mean outflow time of distilled water under the same conditions as prevailed in the estimation of  $s_0$  (that is, the same viscosimeter, temperature, and volume). In Table I are given the results obtained which are represented graphically in Text-fig. 1, where a smooth curve is drawn through the points  $(\rho, \phi)$ .

The validity of employing the 3 per cent substrate solution for estimation of amylase concentration is indicated by the following experiment. Four digestion mixtures were simultaneously observed, employing 3 per cent starch as substrate, in which the enzyme concentrations were in the proportion  $1:\frac{1}{2}:(\frac{1}{2})^2:(\frac{1}{2})^3$ , the greatest concentration being about 4 units ( $Q = 4$ ). The results of five such experiments, with digestions followed in duplicate in each instance, are given in Table II. In this table the means of the duplicate digestion estimates of  $T$  are designated by  $\alpha, \beta, \gamma$ , and  $\delta$  respectively,  $\alpha$  being that for the strongest, and so on in descending order,  $\beta$  for  $\frac{1}{2}$ ,  $\gamma$  for  $\frac{1}{4}$ , and  $\delta$  for  $\frac{1}{8}$  of this strength.

TABLE I

Starch concentration	$\rho$	$\phi$	a. d.
<i>gm./100 cc.</i>			
7.000	2.89	0.800	
6.000	2.46	0.807	0.001
3.000	1.56	0.842	0.001
1.500	1.25	0.890	0.002
0.750	1.12	0.924	0.002
0.375	1.06	0.959	0.004

a. d. is the mean of numerical deviations of  $\phi$  estimated from their mean.

In order to compare these various experiments we define the number  $J$  in each instance by the relation

$$(4) \quad J = \frac{8\alpha + 4\beta + 2\gamma + \delta}{8}$$

and we define

$$(5) \quad \alpha' = \frac{\alpha}{J}, \beta' = \frac{\beta}{J}, \gamma' = \frac{\gamma}{J}, \text{ and } \delta' = \frac{\delta}{J}.$$

The value of these variables will be found in Table III together with their means, dispersion measures (a.d. and A.D.), and  $\Delta$ , the deviation of the mean from its ideal value for exact holding of the reciprocal relation between  $T$  and the active amylase concentration.

It is readily observed that starch solutions prepared from different lots of starch, even though the solutions are the same concentration

TABLE II

Approximate value of $Q$	I		II	III	IV	V
4	$\alpha$	0.242	0.260	0.257	0.230	0.255
		0.129	0.260	0.257	0.248	0.268
		0.186	0.260	0.257	0.239	0.262
2	$\beta$	0.501	0.332	0.528	0.508	0.544
		0.504	0.341	0.526	0.495	0.550
		0.503	0.337	0.527	0.502	0.547
1	$\gamma$	0.99	1.07	1.21	1.03	1.24
		1.01	0.98	1.14	1.02	1.17
		1.00	1.03	1.18	1.03	1.21
$\frac{1}{2}$	$\delta$	2.12	1.95	2.06	2.04	2.13
		2.04	1.96	2.07	1.99	2.19
		2.08	1.96	2.07	2.02	2.16
	$J$	0.948	0.931	1.08	1.00	1.11

TABLE III

Experiment	$\alpha'$	$\beta'$	$\gamma'$	$\delta'$
I	0.196	0.533	1.06	2.19
II	0.279	0.326*	1.11	2.11
III	0.238	0.486	1.19*	1.92
IV	0.239	0.502	1.03	2.02
V	0.236	0.493	1.03	1.95
Mean	0.238	0.504	1.058	2.038
a. d.	0.017	0.015	0.028	0.090
A. D.	0.008	0.008	0.014	0.040
$\Delta$	-0.012	0.004	0.058	0.038

\* Rejected by rule of deviation in excess of 4 (a. d.) of remaining observations from their mean.

by weight, may differ greatly in both viscosity and as media for the viscosimetric estimation of enzyme concentration. The following



experiments were performed in an attempt to find some basis of comparison of results which might so be obtained.

In the same manner as solutions of different concentrations of the standard lot were compared with a 3 per cent dilution, so 3 per cent solutions of different lots were compared with this same standard, and the appropriate values of  $\phi$  estimated, together with  $\rho$  in each instance. The results so obtained are given in Table IV and the points ( $\rho$ ,  $\phi$ ) represented graphically in Text-fig. 1.

TABLE IV

Starch lot	$\rho$	$\phi$
I	1.35	0.932
II	1.41	0.898
III	1.49	0.863
IV	1.56	0.842
V	1.70	0.800
VI	2.51	0.582
VII	3.47	0.43

## DISCUSSION

It is remarkable that the data of Table IV admit of representation of  $\phi$  as a single valued decreasing function of  $\rho$  by a smooth continuous curve as illustrated in Text-fig. 1. It is obvious that we should fail to obtain equivalent digestions merely by restricting variations in  $\rho$  (e.g., by taking a concentration of starch such that  $\rho = 2.5$ ). For the values of  $\phi$  for Lots IV and VI at this point would be approximately 0.8 and 0.6, respectively, whence the ordinary procedure of taking  $T$  as the time required for a 20 per cent change in viscosity obviously would be more than twice as great in the first than in the second case (the shape of the actual digestion curves being concave upward as is well known). Further investigations of other relations between  $\rho$  and  $\phi$  for different lots of starch are in progress in this laboratory. It appears that with the standard lot of starch used as the basis of comparison in these studies, that a 3 per cent solution may be used as an alternative to the 7 per cent solution previously employed, provided the value  $\phi = 0.842$  is substituted for that of  $\phi = 0.800$ . Here also, further work is being undertaken in order to estimate the relative

merits of such systems. The limitations to following digestions only in duplicate (two viscosimeters) was admitted in certain of these experiments only because of a desire to run four digestions simultaneously, and a limitation under then existing circumstances to the simultaneous employment of not more than eight viscosimeters. In Table III two rejections have been made in the usual manner.

#### SUMMARY

The results of employing different concentrations of starch and starch from different lots as substrates in the viscosimetric estimation of amylase concentration have been compared with the object of ultimate attainment of reproducible standards, independent of successive comparison.



## A REPRODUCIBLE STANDARD SUBSTRATE IN STARCH-AMYLASE VISCOSIMETRY

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(Accepted for publication, July 20, 1932)

A viscosimetric method of estimating enzyme concentration with special reference to amylase has been discussed in another communication<sup>1</sup> from this laboratory; and, in a later report,<sup>2</sup> the effects of variation in the concentration of starch from the standard lot and variation in lot of prepared soluble starch have been contrasted, indicating that only within a small range of variation of  $\rho$  (defined there as the relative viscosity of the blank with respect to water) may comparability of digestion rates be expected. These experiments were completed in June, 1930, but were not published at that time. The object was to demonstrate some of the difficulties (a subject treated also by Chesley)<sup>3</sup> which may be encountered in attempts to compare results with different lots of soluble starch in the hope that some method of obtaining reproducible standard substrate solutions might be suggested. In the present communication we propose to outline a method of approximating the 3 per cent starch solution previously suggested as a standard<sup>2</sup> by use of a composite of other lots of soluble starch.

The experimental procedure involved simply the mixing of soluble starch of two *original* lots (one yielding a more viscous and the other a less viscous solution than the standard, if preparation was made in standard manner<sup>1,2</sup>) in such proportion as to give a composite approximating the standard with respect to the value of  $\rho$  obtained from the conventional *blank*, 1 part of 0.85 per cent NaCl added to 5 parts by volume of a 3 per cent starch solution prepared in the standard manner, at  $37.50 \pm 0.03^\circ\text{C}$ .

<sup>1</sup> Thompson, W. R., Johnson, C. E., and Hussey, R., *J. Gen. Physiol.*, 1931-32, **15**, 1.

<sup>2</sup> Wies, C. H., and McGarvey, S. M., *J. Gen. Physiol.*, 1932-33, **16**, 221.

<sup>3</sup> Chesley, L. C., *J. Biol. Chem.*, 1931, **92**, 171.

In the previous work<sup>2</sup> all available lots of soluble starch have been enumerated (from I to VII) in the order of increasing values of  $\rho$  so obtained, and it so happened that the lot previously chosen as a standard<sup>1</sup> was No. IV, the median with three others on each side. Thus there were exactly  $3 \times 3 = 9$  possible combinations of pairs of lots in this sequence available for preparation of composites of the above mentioned type. These were prepared ( $\rho$  approximating the ideal value, 1.562) and in each case digestions with the same amylase concentration in a composite and a standard lot 3 per cent substrate were observed in quadruplicate as previously described<sup>1,2</sup> in order to obtain a ratio ( $R$ ) of the corresponding mean times required for the

TABLE I

Relative time for attainment of standard change in viscosity with composite starch substrates of the same initial viscosity and total concentration as the standard substrate using the same proportion of amylase in simultaneous digestions with standard and composite.

Relative digestion time for pair composites			
Starch No.	V	VI	VII
III	0.952*	0.933	0.952
II	1.015	0.979	0.747
I	0.951	0.722	0.642

\* a.d. = 0.041

standard change in viscosity,  $s_0 (\phi - 1)$ . The results are given in Table I, that for the composite (III, V) being the mean of four independent observations (a.d. = 0.041). Four such independent observations of comparison in the same manner of two preparations from the standard lot of starch (No. IV) gave the value,  $R = 1.013$  with a.d. = 0.031.

Apparently, the values of  $R$  obtained do not differ significantly from the ideal value of unity except in the three cases in the lower right part of the table consisting of the extreme and near extreme mixtures (I, VII; I, VI; and II, VII). Thus from two *original* lots of soluble starch prepared in as nearly as practicable the same manner as the standard lot but giving in the blank (prepared in standard manner) a higher and

a lower value of  $\rho$ , respectively, we can prepare a composite giving to close approximation (say within 0.005) the standard value of  $\rho = 1.562$  in the *blank* and use this composite in place of the standard lot provided that the original values of  $\rho$  are within the indicated acceptable ranges (1.41 to 1.562 and 1.562 to 2.51). Then the digestion data obtained with such a composite may be expected to compare favorably with similar data obtained with the standard lot, IV. Accordingly, we have at least a crudely reproducible standard (independent of successive comparison) which may be used to furnish a common basis of comparison of results obtained in different laboratories, which has been lacking. Suggestions in this regard made by other writers have been shown to be not nearly as satisfactory in the work of the previous report<sup>2</sup> as well as in comparisons made by Chesley.<sup>3</sup>



## SIMILARITY OF THE KINETICS OF INVERTASE ACTION IN VIVO AND IN VITRO. II

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Although very exhaustive studies have been made of the kinetics of the action of enzymes *in vitro*, by the use of active cell extracts, there has been serious doubt as to whether these results give a true picture of the manner in which the enzyme accomplishes its physiological function; *i.e.*, its activity *in vivo*. This doubt has recently been expressed in the opinion of Linderstrøm-Lang and Holter (1) that "it is actually only in the study of the action of typical secretion enzymes, outside of cells, that experiments *in vitro* may be considered with any certainty to reproduce the processes taking place in the organism." That this is a somewhat pessimistic viewpoint has been shown by recent work of the authors (2), in which they were able to demonstrate that the course of hydrolysis of sucrose by invertase, a typically intracellular enzyme, could be followed with equal precision irrespective of whether the enzyme was still in the living cells or whether it had been extracted by means of autolysis. Furthermore, it was found that the inversion of sucrose when caused by the physiological action of the living cells followed a course identical with that obtained when inversion was caused by action of cell-free solutions of the enzyme.

Inasmuch as this identity indicates that the kinetics of a single reaction, namely, the hydrolysis of sucrose, may be quantitatively measured *in vivo* despite the complexity of the protoplasmic environment, it was decided to investigate in the living cells certain other kinetic relationships which have previously been shown to characterize the behavior of invertase solutions. Possibly the most widely applicable kinetic relationship in the study of enzymes is the dependence of their reaction velocity on the pH of their reaction medium. Michaelis and coworkers (3, 4) have found that the curve obtained when the relative velocities of enzymic hydrolysis of sucrose are plotted



against the pH values at which the velocities were measured, resembled a typical dissociation-residue curve. In subsequent years this phenomenon has appeared to be characteristic of enzymes in general and has been a means of demonstrating the different optimal pH zones of various enzymes.

Because of the lack of definite information about the conditions of hydrogen ion concentration and buffering power existing in the living cell any interpretation of the pH-activity relationships of an intracellular enzyme must be made by comparison with the relationships found when an enzyme solution extracted from the same organism is used. The necessity for using a method of comparison for interpreting the physiological activity of an intracellular enzyme is shown by Quastel's (5) criticism of the work of Mann and Woolf (6) in which they investigated the effect of the changes of salt and of hydrogen ion concentration upon the activity of fumarase in the cells of *B. coli*. His objections to any interpretation of their results is based upon their inability to obtain an active solution of fumarase with which to compare their results obtained *in vivo*. Since yeast invertase can be obtained in the form of a fairly stable solution it lends itself admirably to this kind of comparative study.

The present paper presents a comparison of the pH activity relationships of yeast invertase as evidenced by the living cells and by the enzyme extracted from them by autolysis.

#### EXPERIMENTAL

##### *Hydrolysis of Sucrose by Invertase Solution at Various pH Values*

The invertase preparation used was prepared by autolyzing *Saccharomyces cerevisiae* with toluene and water. The invertase was precipitated from the autolysate in 50 per cent ethyl alcohol and extracted from this precipitate with water. In determining the velocities of hydrolysis, 25 cc. of the enzyme solution were pipetted into 100 cc. of 12.5 per cent sucrose solution containing 0.05 M citrate buffer at the desired pH and at  $25^{\circ} \pm 0.01^{\circ}\text{C}$ . At recorded time intervals 25 cc. samples of the hydrolyzing solution were removed and to each sample was added a drop of sodium hydroxide solution of sufficient strength to raise the pH to 8-9 at which pH hydrolysis ceases. After allowing time for mutarotation to occur the samples were polarized at  $25^{\circ}\text{C}$ . in a 2 dm. tube using a mercury arc light of wave length  $546.1\mu\mu$ . The pH of the various hydrolyzing solutions was determined electrometrically by the use of the hydrogen electrode.

The data from the experiments in which invertase solutions were used are presented in Table I.

TABLE I  
*Variation of Hydrolytic Activity of Invertase Solutions with pH*

pH	$\Delta T^*$	Rotation	$\Delta$ Rotation	Change per min. (mean value)	Recalculated velocity†	Relative velocity
	<i>min.</i>	<i>degrees</i>		<i>degrees</i>		<i>per cent</i>
2.71	0	15.31				
	10	14.80	0.51			
	20	14.31	1.00	0.0505	0.0533	90.0
3.19	0	15.31				
	10	14.79	0.52			
	20	14.21	1.10	0.0535	0.0564	95.3
3.74	0	15.17				
	10	14.61	0.56			
	20	14.06	1.11	0.0558	0.0589	99.5
4.08	0	15.20				
	10	14.65	0.55			
	20	14.06	1.14	0.0560	0.0591	99.8
4.67	0	15.13				
	10	14.59	0.54			
	20	14.04	1.09	0.0543	0.0573	96.8
5.01	0	15.23				
	10	14.66	0.57			
	20	14.09	1.14	0.0570	—	96.3
5.69	0	14.66				
	10	14.14	0.52			
	20	13.65	1.01	0.0513	—	86.7
6.04	0	15.42				
	10	14.96	0.46			
	20	14.48	0.94	0.0465	—	78.5
6.44	0	15.42				
	10	15.01	0.41			
	20	14.61	0.81	0.0408	—	68.9
7.05	0	15.49				
	10	15.22	0.27			
	20	14.96	0.53	0.0268	—	45.3
7.44	0	15.51				
	10	15.36	0.15			
	20	15.21	0.30	0.0150	—	25.3

\* The initial samples were taken 3 minutes after the start of the hydrolyses. An exception was the case of the hydrolysis at pH 5.69 where the initial sample was taken 13 minutes after the start of the hydrolysis.

† Since the hydrolyses were divided between 2 successive days and a fresh enzyme solution was used the 2nd day, it was necessary to recalculate the velocities from pH 2.71–4.67 to the basis of the remaining hydrolyses.

Recalculated velocity = observed velocity  $\times$  1.055.

### *Hydrolysis of Sucrose by Living Cells of S. Cerevisiae at Various pH Values*

In these hydrolyses a water suspension was made of washed, pressed yeast. 25 cc. of this suspension were pipetted into 100 cc. of 12.5 per cent sucrose solution containing 0.05 M citrate buffer at the desired pH. The hydrolyzing mixtures were shaken continuously in a thermostat at  $25^{\circ} \pm 0.01^{\circ}\text{C}$ . 25 cc. samples were removed and inversion stopped as described above and were immediately filtered by suction through porous bottom Gooch crucibles which completely removed the cells. The filtrates were polarized as in the preceding method. The pH values of the reaction media were determined as above on samples removed and filtered cell-free at times midway between the taking of the two samples for polarization.

These data are given in Table II, *a* and *b*.

The results of two series of experiments in which living cells were used are included because preliminary work showed that it was best to use one-sample hydrolyses. It was found that on the alkaline side of the optimum, as the hydrolyses progressed beyond the time of taking the second sample, fermentation became a complicating factor and the pH of the hydrolyzing mixtures dropped toward the optimum while in the most acid hydrolyses the pH of the mixture was found to increase slightly.

For the purpose of determining whether or not there was any death of cells due to the extreme variation of pH to which they were subjected, it was necessary to use an independent experiment, since the hydrolyses were not performed under known sterile conditions. The pH of 3 aliquot portions of a yeast suspension was adjusted to 2.60, 4.83, and 7.25 in the presence of 0.05 M citrate buffer. After standing 1 hour 1 cc. of each of these suspensions was diluted one million times and 1 cc. samples of these diluted suspensions were plated out on Petri dishes in a sucrose-malt-agar medium at pH 4.70. Since after incubation at room temperature for 48 hours the colony counts, made of the three groups of plates, did not differ by more than 4 per cent, the contention that exposure of the yeast cells to the conditions of hydrogen ion concentration used in these experiments causes no appreciable mortality is justified. The general technique used in this experiment was the one advocated by the American Public Health Association in the 1925 edition of Standard methods of water analysis.

In order that the data in Tables I and II may be compared, all

TABLE II, *a*  
*Variation of Hydrolytic Activity of Yeast Suspensions with pH*

pH	$\Delta T^*$	Rotation	$\Delta$ Rotation	Change per min.	Relative velocity
	<i>min.</i>	<i>degrees</i>		<i>degrees</i>	<i>per cent</i>
2.65	0	15.34			
	15	14.77	0.57	0.0380	84.4
3.21	0	15.32			
	15	14.70	0.62	0.0413	91.8
3.69	0	15.20			
	15	14.55	0.65	0.0433	96.2
4.12	0	15.18			
	15	14.51	0.67	0.0447	99.3
4.67	0	15.13			
	15	14.46	0.67	0.0447	99.3
4.99	0	15.19			
	15	14.54	0.65	0.0433	96.2
5.68	0	15.11			
	15	14.50	0.61	0.0407	90.4
6.04	0	15.36			
	15	14.81	0.55	0.0367	81.6
6.45	0	15.33			
	15	14.86	0.47	0.0313	69.6
7.00	0	15.48			
	15	15.16	0.32	0.0213	47.3
7.26	0	15.49			
	15	15.23	0.26	0.0173	38.4

TABLE II, *b*

pH	$\Delta T^*$	Rotation	$\Delta$ Rotation	Change per min.	Recalculated velocity†	Relative velocity
	<i>min.</i>	<i>degrees</i>		<i>degrees</i>		<i>per cent</i>
2.65	0	15.36	0.53	0.0353	0.0402	85.2
	15	14.83				
3.14	0	15.31	0.56	0.0373	0.0424	89.8
	15	14.75				
3.75	0	15.21	0.60	0.0400	0.0455	96.4
	15	14.61				
4.07	0	15.22	0.62	0.0413	0.0470	99.6
	15	14.60				
4.65	0	15.24	0.62	0.0413	0.0470	99.6
	15	14.62				
5.00	0	15.27	0.91	0.0455	—	96.4
	20	14.36				
5.67	0	15.20	0.65	0.0433	—	91.7
	15	14.55				
6.02	0	15.43	0.62	0.0413	—	87.5
	15	14.81				
6.45	0	15.42	0.53	0.0353	—	74.8
	15	14.89				
6.93	0	15.50	0.37	0.0247	—	52.3
	15	15.13				
7.22	0	15.51	0.24	0.0160	—	33.9
	15	15.27				

\* The initial samples were taken 5 minutes after the start of the hydrolysis.

† See footnote at end of Table I.

Recalculated velocity = observed velocity  $\times$  1.138.

velocities have been computed as fractions of the maximum velocity in each series of hydrolyses and these fractional or relative velocities are plotted against pH in Fig. 1. From this graph it can readily be

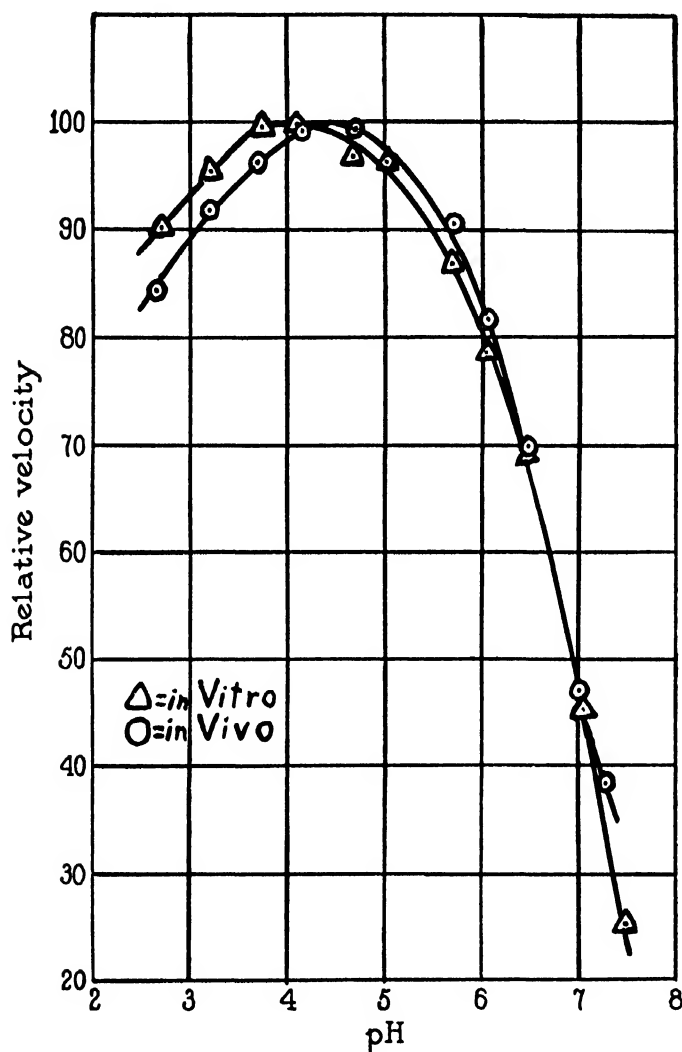


FIG. 1

seen that the pH dependence of invertase in living cells is practically the same as that formerly observed with the enzyme solution extracted from the cells and that the acid dissociation constant of the enzyme, which is equal to the hydrogen ion concentration at half the maximum

velocity, is identical in the two cases. The value of this constant is found by inspection of Fig. 1 to be  $10^{-6.95}$  for this strain of *S. cerevisiae*.

#### DISCUSSION

An intracellular enzyme is one which is not secreted from the live cell in which it is produced into the medium surrounding it; that is, it displays its activity inside the live cell. It is the general opinion (7, 8) of workers in this field that invertase is an intracellular enzyme. To say that an enzyme is intracellular does not accurately enough define the region in which it works physiologically. If we think of a yeast cell as conforming to the following accepted morphological ideas, that is, that it consists of a central fluid, the cell sap, surrounded by a cytoplasmic layer of high protein content and bound externally by a cellulosic cell wall, we define at least four distinct regions in which the enzyme may act: in the cell sap, in the cytoplasmic layer, on the external surface of the cytoplasm, or on the surfaces of the cell wall.

Small (9), in his recent complete compilation of data pertaining to the rôle of hydrogen ion concentration in plant cells, contends that the pH of the cytoplasm lies between 5.2 and 6.2 with little or no variation beyond these limits in the living state. Because of its high protein content it is an efficient buffer, and can exist in the live state in contact with solutions ranging in pH from 2 to 10. The cell sap, a much less efficient buffer, would constantly be much more acid than the cytoplasm because of constant metabolic acid production. On this basis Small postulates that enzymes which exhibit maximum activity at a pH lower than 5.2, including invertase, are secreted from the cytoplasm into the cell sap, while those whose pH optima lie between 5.2 and 6.2 act in the cytoplasm itself. In contrast to this hypothesis, Haldane (10) says, "the yeast cell contains enzymes with pH optima varying from pH 4.6 to about 8 and most of them must work at a non-optimal pH." These differing views, because of lack of direct experimental evidence, are purely speculative. In view of the limited knowledge of the general internal environmental conditions and activity of cells, we shall refrain from making any definite claim as to the region in which sucroclastic activity is displayed. However, the results of the present investigation show that the invertase in living

yeast cells, in whatever region it may be, is as freely exposed to changes in hydrogen ion concentration of the external environment as is the enzyme after extraction from the cells. Because of the immediate response of the intracellular invertase to changing hydrogen ion concentration, the suggestion is made that this enzyme may function only in the outer regions of the cell, possibly, in view of the protein-like nature of invertase, at the external surface of the cytoplasm.

Another observation may be made with regard to Weidenhagen's (11) new theory of the specificity of carbohydrase action. This theory postulates that  $\alpha$ -glucosidase, *e.g.* maltase, hydrolyzes sucrose at pH 6.9 with about the same velocity with which it hydrolyzes maltose at the same pH. Since the yeast used in the present investigation contained considerable maltase, while the invertase solution contained none, one would have expected, on the basis of Weidenhagen's theory, to obtain a distorted pH-activity curve between pH 6 and 7.5 when the live cells were used due to the increasing sucroclastic activity of the maltase. However, the coincidence of the two experimental curves throughout this range of pH indicates that there was no perceptible hydrolysis of sucrose by maltase.

#### SUMMARY

1. The pH-activity relationship of invertase has been studied *in vivo* and *in vitro* under identical external environmental conditions.
2. The effect of changing ( $H^+$ ) upon the sucroclastic activity of living cells of *S. cerevisiae* and of invertase solutions obtained therefrom has been found, within experimental error, to be identical.
3. The region of living yeast cells in which invertase exerts its physiological activity changes its pH freely and to the same extent as that of the suspending medium. It is suggested that this may indicate that this intracellular enzyme may perform its work somewhere in the outer region of the cell.
4. In using live cells containing maltase, no evidence of increased sucroclastic activity around pH 6.9, due to the action of Weidenhagen's  $\alpha$ -glucosidase (maltase), was found.

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# FACTORS INVOLVED IN THE USE OF ORGANIC SOLVENTS AS PRECIPITATING AND DRYING AGENTS OF IMMUNE SERA

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In a preliminary report (1) we have given a method by which immune serum can be prepared in dry form by precipitation at room temperature with acetone followed by ether extraction. Since the work of Mellanby reported in 1907 (2), it appears to have been accepted that precipitation of serum proteins by ethyl alcohol or acetone at a temperature above 14°C. results in varying degrees of denaturation of the serum proteins. Mellanby found that when precipitation is carried out at temperatures below 14°C. the precipitate is completely resolvable. He designated this temperature the critical temperature. In addition to being a dividing point between denaturation and no alteration of solubility of the proteins, it was also the point of minimum precipitation, any concentration of alcohol exerting least precipitating effect at this temperature. Mellanby (3) later (1908) extended his work to diphtheria antitoxin and found antitoxic value was not affected by alcohol as long as no coagulation occurred. Felton (4) recently reported similar findings. Later work by Hardy and Gardiner (5) and Hartley (6) has shown that the destructive effect of alcohol and acetone upon antibodies is related to the denaturation of the serum proteins. From the work of Mellanby these later workers devised methods by which serum proteins could be precipitated in the cold and subsequently extracted with ether, dried, and a powder obtained. This material was completely soluble and possessed its original antibody activity excepting that precipitating action on specific antigen was diminished or lost (6). These workers have attributed lack of denaturation to the low temperature.

Mellanby did not report the effect of ethyl alcohol in concentrations above 75 per cent. The effect of temperature, hydrogen ion concentration, and of time in these higher concentrations has apparently not been reported. As we have previously noted (1) there exists a critical concentration of the organic solvents, ethyl alcohol, and acetone in the zone of 70 to 75 per cent concentration at which the coagulating effect is maximal. As the concentration is increased from this point there is progressively less coagulation until at concentrations exceeding about 87 per cent precipitation can be accomplished at room temperature and yet the proteins remain completely resolvable. The following report deals with our studies of this phenomenon and its relationship to known facts concerning the action of organic solvents upon serum proteins and antibodies.

*Relation of Concentration of Organic Solvents to Precipitation of Serum Proteins and Resolubility of the Precipitate*

In order to determine the rôle of concentration of the organic solvents studied, two general methods have been employed. In the first of these methods, the organic solvent in increasing quantities was added to a constant volume of a given dilution of serum. This gave increasing percentage concentration of the solvent but since the volume increased also the serum concentration became progressively less. The results of one such experiment are shown in Fig. 1. Working at room temperature (22°C.) horse serum was diluted 1:5 with 0.85 per cent NaCl solution and 0.5 cc. added to each of eleven tubes. Absolute ethyl alcohol was then added quickly followed by immediate shaking. The precipitate was approximated after 5 minutes; all tubes were then centrifuged, and the supernatant decanted. The solubility of the precipitates was approximated by adding 2 cc. of saline to each tube followed by immediate shaking.

It will be noted that precipitation was complete in concentrations of 60 per cent<sup>1</sup> or above and that precipitates formed between 60 to 70 per cent concentration was least soluble while precipitates formed in the presence of greater than 87 per cent alcohol were completely resolvable. The agglutinin loss, when agglutinating serum was employed, approximately paralleled the loss in solubility.

<sup>1</sup> All reference to concentration used in this report refers to final concentration of the organic solvent in the mixture.

Sufficient alcohol was added to each of the decanted supernatants to bring the total volume to 5.0 cc. The precipitates were centrifuged down and the solubility determined as before. It was noted that the proteins not precipitated by lower concentrations of alcohol are completely resolvable after precipitation in the presence of 90 per cent alcohol.

Certain precautions are necessary in order to thus demonstrate the rôle of concentration upon precipitation and resolubility. Even very brief exposure of the serum to the action of 60 to 70 per cent alcohol at room temperature results in considerable loss in solubility. Therefore when precipitation is accomplished in high concentrations of

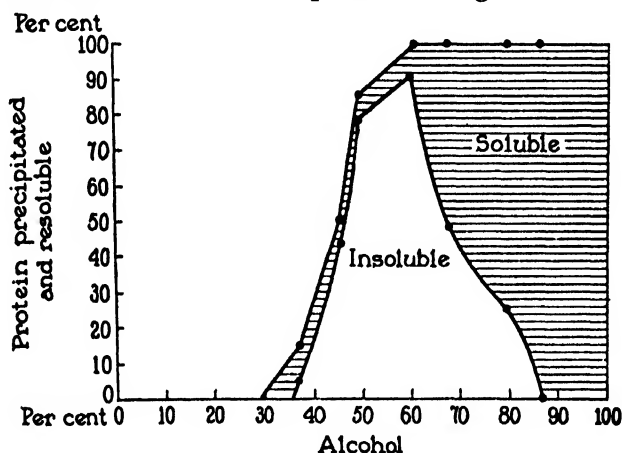


FIG. 1. Alcohol concentration and precipitation and resolubility of serum proteins. Serum diluted 1:5, plus increasing amounts of alcohol. Temperature 22°C. Duration of exposure 5 minutes.

alcohol the mixing must be instantaneous. Slowly raising the concentration through 60 to 70 per cent up to 90 per cent results in some loss in solubility. Furthermore, the solubility of precipitates formed in 75 per cent alcohol is not enhanced by subsequent extraction with 95 per cent absolute alcohol. Similarly if precipitation is accomplished in 90 per cent alcohol and the concentration lowered to 70 per cent by adding water there is a loss in solubility, the resulting precipitate being equally as insoluble as if precipitation had been accomplished in 70 per cent alcohol. Hence in determining solubility of the precipitate immediate shaking is necessary. If saline is carefully added to the precipitate of a tube containing 90 per cent alcohol,

and allowed to stand for 30 minutes the precipitate does not dissolve and upon subsequent shaking the solution is incomplete. If shaken immediately after the addition of the saline the precipitate completely dissolves.

The second general method that has been used for determining the rôle of the concentration of the organic solvent was to employ constant volumes of solvent-water mixture to give the desired concentration of solvent after the addition of serum. By this method all factors could be kept constant and the effect of varying concentration of the organic solvent more accurately determined. For example 0.25 cc. of serum was added to 4.75 cc. of alcohol-water mixtures of such proportions that the final alcohol concentration was 10, 20, 30, etc., per cent. The results by this method were essentially the same as in the experiments discussed above. As will be shown below some variation in the degree of precipitation by any given alcohol or acetone concentration is introduced by varying the concentration of the serum. All subsequent results are on determinations made employing constant volumes and constant serum concentration for any one set-up.

By either method ethyl, methyl, and propyl alcohols gave practically the same results. Acetone in general was found to be a slightly better precipitating agent and at the same time the precipitates formed were slightly more soluble. The critical concentration, or point of greatest loss of solubility was in the same range with all four solvents.

#### *Rôle of Temperature and Duration of Exposure upon Precipitation and Resolubility of the Precipitate*

Mellanby (2) has shown that up to 70 per cent concentration of ethyl alcohol the precipitating effect and coagulating effect of ethyl alcohol increases rapidly during the first few minutes of exposure and more slowly as the duration is prolonged. At all temperatures he reports having investigated, complete precipitation occurred immediately in 70 per cent alcohol.

The influence of duration of exposure to 95 per cent ethyl alcohol at various temperatures upon the resolubility of the precipitate is shown in Table I. These results were obtained by adding 0.25 cc. of beef serum to 4.75 cc. of absolute ethyl alcohol, both components being at the temperatures indicated before mixing.

The duration of exposure was the time elapsing after mixing before centrifuging so that the actual time elapsing between mixing and dilution with water to determine solubility was in each case about 4 minutes more than the figures would indicate. All determinations were centrifuged at room temperature, and solubility determined by adding to each tube 2.5 cc. of distilled water followed by immediate shaking.

The results indicate that there is no loss in solubility following precipitation by and exposure to 95 per cent alcohol for 24 hours at

TABLE I

*Influence of Duration of Exposures to 95 Per Cent Alcohol at Various Temperatures upon Resolubility of the Precipitated Serum Protein*

Duration of exposure	Temperature			
	5°C.	25°C.	35°C.	50°C.
1 min.	C.s.	C.s.	C.s.	P.s.
10 min.	C.s.	C.s.	C.s.	Ca. 75 per cent
60 min.	C.s.	C.s.	Slight precipitate left	P.s.
4 hrs.	C.s.	C.s.	More precipitate left	P.s.
24 hrs.	C.s.	Very slight flocculent precipitate undissolved	More precipitate left	P.s. Ca. 25 per cent

C.s. = completely soluble

P.s. = partially soluble

5°C. There is beginning loss in solubility in 24 hours at 25°C., in 60 minutes at 35°C., and immediately at 50°C.

The effect of temperature on precipitation and resolubility at various alcohol concentrations is shown graphically in Fig. 2. Again 0.25 cc. of beef serum was added to 4.75 cc. of alcohol-water mixture in such proportions that the final alcohol concentration was 10, 20, 30, etc., per cent up to 95 per cent. All components were brought to the desired temperature before mixing. After 30 minutes the degree of precipitation was approximated, the tubes centrifuged, and the solubility of the precipitates determined in each case in 2.5 cc. of distilled water. The 5°C. determinations were carried out in their entirety in

the cold room at 5°C. The shaded area indicates the approximate amount of the precipitated material that was resolvable.

On the first graph (upper left) is given the comparative precipitations at 5°, 22°, 37°, and 50°C. together with the resolubility of the 50°C. precipitate. It will be seen that precipitation occurs in definitely lower concentrations of alcohol at 5°C. the precipitating effectiveness decreasing as the temperature is raised. The difference

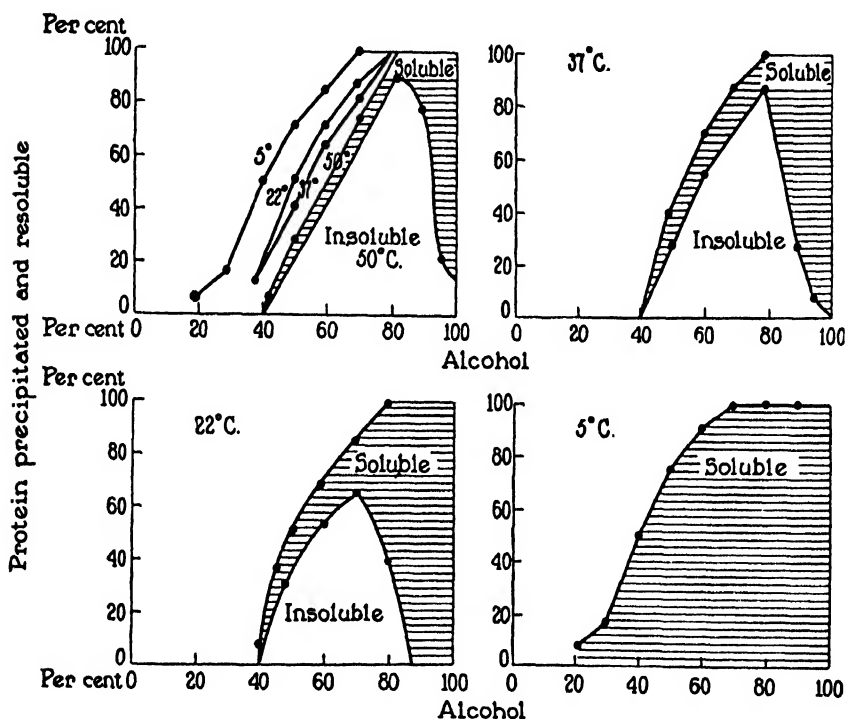


FIG. 2. Effect of temperature on precipitation and resolubility. Alcohol and beef serum.

between the three higher temperatures is not so pronounced but nevertheless is present. This is contrary to the findings of Mellanby (2) who found minimum precipitating effect occurred at 14°C. and that precipitating effect increased as the temperature was increased.

The graphs show clearly the effect of temperature upon resolubility. At 5°C. precipitation is greatest and the precipitate produced by all concentrations of alcohol were soluble. There was definite loss in solubility in concentrations up to 95 per cent at 37°C. and 50°C.

The difference in the 20°C. curves here shown and that in Fig. 1 is attributed to the difference in the concentration of the serum proteins in the two cases and the duration of exposures. As previously stated the precipitating effectiveness of these solvents increases with increasing concentration of the serum proteins and with increasing duration of exposure.

These same experiments have all been repeated using acetone in place of ethyl alcohol with essentially the same results. Slight differences were encountered due to the fact that acetone is a slightly better precipitating agent and also the acetone precipitates tend to be a little more soluble. The general phenomena, however, appear to be the same in the two cases.

*Rôle of Hydrogen Ion Concentration upon Precipitation and Resolubility of the Precipitate*

In the preceding experiments the hydrogen ion concentration of the serum has not been changed. Mellanby (2) observed that slight acidification of serum rendered it more readily precipitated by alcohol, while greater acidification decreased precipitation. It is generally stated that alcohol precipitation is inhibited by acids and alkalis.

In Fig. 3 is given a comparison of precipitation and resolubility of the precipitate by various alcohol concentrations at pH 6.0 and 7.5. Precipitation at pH 6.0 is as good in 20 per cent as in 40 per cent alcohol at pH 7.5. The precipitates show little variation in solubility, both being completely soluble where precipitation occurred in concentrations of alcohol exceeding 87 per cent.

The effect of pH upon precipitation is more clearly shown in Fig. 4 in which pH is plotted as abscissa against per cent of proteins present which are precipitated as ordinates. The results for both ethyl alcohol and acetone, each at various concentrations, are shown. A sample of serum was adjusted to each of the desired pH values and then an equal portion of 0.25 cc. added to a series of tubes each containing 4.75 cc. of organic solvents so diluted that the final concentrations would range from 10 to 95 per cent. The results were read on a basis of the approximate per cent of proteins precipitated after 30 minutes. The precipitates were then thrown down by centrifuging and the solubilities determined in 2.5 cc. of distilled water.



It will be seen from the curves that maximum precipitation occurs in the range of pH 5.0 to 6.0. Ethyl alcohol caused no precipitation in any concentration at pH 4.2 and only slight precipitation in high

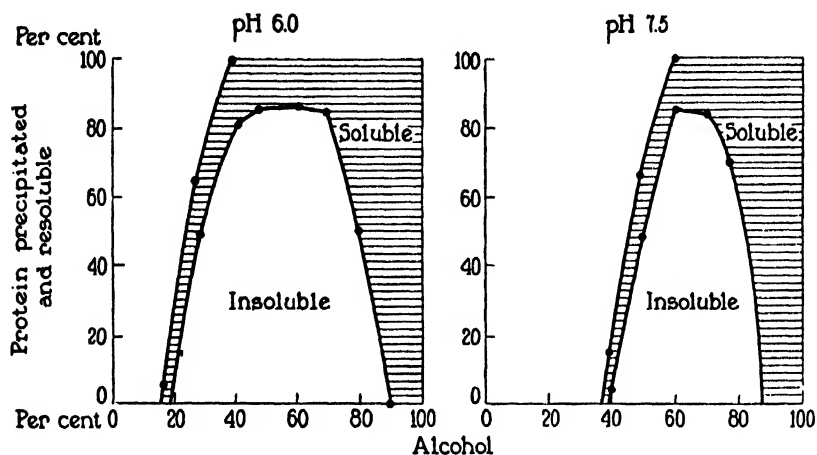


FIG. 3. Comparison of precipitation of serum protein at pH 6.0 and 7.5. Re-solubility of precipitate.

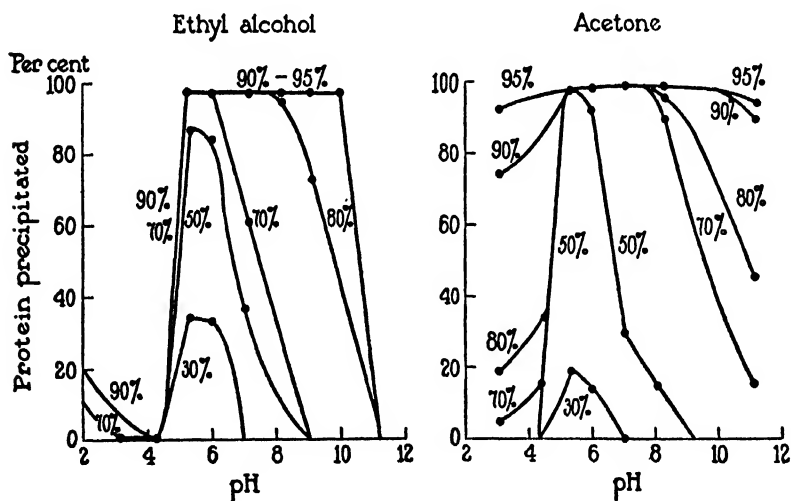


FIG. 4. Relation of pH and precipitation of serum proteins by ethyl alcohol and acetone.

alcohol concentrations in the more acid range. As the alkalinity increases beyond pH 6.0 the amount of precipitation decreases, there being no precipitate in concentrations of less than 70 per cent

beyond pH 9.0. As the concentration of alcohol is increased the pH range through which precipitation occurs becomes broadened.

The same features are found in acetone precipitation, except that acetone is a more active precipitating agent and especially in the acid range precipitation is more complete. In lower concentration of acetone (*e.g.*, 30 to 50 per cent) the zone of maximum precipitation is the same as for ethyl alcohol.

The solubilities of the precipitates are not shown in the figure. The zone of maximum loss of solubility (critical concentration) is evident regardless of the pH but in general the solubility of the precipitates produced by high concentrations of these solvents becomes progressively less with changes in hydrogen ion concentration either way from pH 6.0.

In the above experiments the serum was adjusted to a specified pH prior to adding the alcohol or acetone. Quite different results are obtained if the acid or alkali is added to the organic solvent and the unadjusted serum added. There is uniformly more precipitate produced under the latter conditions. Furthermore, the precipitate is less soluble. If unadjusted serum is added to the alcohol or acetone and then acid or alkali is added to the mixture there is partial resolution of the precipitate, the end result being almost identical with results obtained when the solvent is acidulated or alkalinized prior to the addition of the serum.

#### *Effect of the Concentration of the Serum upon Precipitation of Serum Proteins by Organic Solvents*

It was indicated above that the serum concentration very materially alters the alcohol precipitation curve. With increasing concentrations of serum a lower concentration of alcohol has been found necessary to yield a given percentage of the proteins precipitated.

This phenomenon has been studied in its relation to precipitation of serum proteins by acetone at 5°C. Felton (4) has suggested that some fractionation of serum proteins by precipitation with acetone in the cold is possible. The rôle of serum concentration in such fractionation has been investigated with the results given in Table II.

In each of these experiments the serum was added in the desired dilutions to a test-tube and acetone added to give the indicated final

concentrations. The results are given in terms of approximate percentage of the total proteins present that are precipitated assuming 8 plus to be complete precipitation. The plus signs therefore do not indicate the absolute amount of precipitate. 4 plus precipitation almost quantitatively separates the globulins and albumins, the globulins being precipitated. It is seen that this is accomplished in 33 per cent, 36 per cent, 40–45 per cent, and 50 per cent in serum undiluted, diluted 1:2, 1:5, and 1:10 respectively.

In these lower concentrations of the organic solvents, then, the concentration of the serum plays an important rôle in determining the proportion of the total proteins present that will be precipitated. Apparently the same phenomenon occurs in the case of ethyl alcohol.

TABLE II

*Influence of Concentration of Serum on Precipitation by Acetone at 5°C.*

(Complete precipitation of protein denoted by 8 plus)

Serum dilution	Acetone						
	25 per cent	30 per cent	33 per cent	36 per cent	40 per cent	45 per cent	50 per cent
Undiluted	+	2+	4+	6+	7+	—	
Dilution 1.2	+	+±	3+	4+	6+	—	
Dilution 1.5	±	+±	—	3+	4+	4+	6+
Dilution 1.10	?	±	+	—	2+	3+	4+

### *Effect of Extraction of Dried Immune Serum by Various Reagents*

Rabbit *B. coli* immune serum in 0.25 cc. portions was precipitated in 4.75 cc. of absolute alcohol, extracted with alcohol followed by three washings with ether, and the resulting precipitate dried at 37°C. These samples of dried serum were used to determine the effect of various organic substances upon first, the subsequent solubility of the serum proteins in water; and second, the effect upon the agglutinin titer. The substance being tested was added directly to the tube containing the dried serum followed by sufficient shaking to thoroughly mix and allowed to stand in most cases for 1 hour. The serum constituents were then centrifuged down, the supernatant poured off, and the residual organic extractant then extracted from the protein mass by two washings with ether. The precipitate was finally dried at

37°C. to drive off the ether and the precipitate suspended in 2.5 cc. of saline. The agglutinin titer of the dissolved or partially dissolved material was then determined.

The following substances were employed with the results given:

1. Amyl alcohol—does not extract antibodies or render proteins insoluble.
2. Chloroform—definite decrease in agglutinin titer and some loss in solubility. No agglutinin activity demonstrable in residue of chloroform extract after evaporation to dryness.
3. Pyridine—no antibody decrease and no loss in solubility.
4. Ethylene dichloride—no antibody decrease and no loss in solubility.
5. Benzene—no antibody decrease and no loss in solubility.
6. Ethyl acetate—no antibody decrease and no loss in solubility.
7. Ethylene glycol—no antibody decrease and no loss in solubility.
8. Aniline—does not extract agglutinins; causes some decrease in solubility and agglutinating activity.
9. Glycerine—proteins completely soluble. No loss in antibody activity. Precipitated from glycerine solutions by alcohol in almost the same alcohol concentrations as from watery solutions.

### *Effect of Alcohol Precipitation and Ether Drying upon the Antibody Titers of Immune Sera*

To each of twelve small test-tubes was added 0.2 cc. of rabbit *B. coli* immune serum followed in each case by 5 cc. of 95 per cent ethyl alcohol. The heavy flocculent precipitates were each washed with 5 cc. of 95 per cent alcohol, 5 cc. of alcohol-ether 1:1, and finally two times with ether. The final precipitate was dried for 1 hour at 37°C.

The tubes of dry powder were stoppered with cotton plugs. Tubes 1-4 inclusive were placed in the incubator (37°C.); Tubes 7 and 8 were left at room temperature; Tubes 9-12 inclusive were placed at 5°C. Tubes 5 and 6 were heated to 165°C. for 1 hour. 2 cc. of saline were then added to each of the latter two tubes. There was only slight solution and complete loss of agglutinating activity.

After 1 week incubation 2 cc. of saline were added to each of Tubes 1, 7, and 9. There was complete solution in each case. The agglutinin titers of each redissolved precipitate was compared with untreated serum which had been kept at 5°C. The agglutinin titers were the same in each case.

After 6 months 2 cc. of saline were added to each of Tubes 4, 8, 11. Solution was not complete in Tubes 4 and 8 (37°C. and room temperature respectively) there remaining a slight flocculent precipitate in each. The solution of Precipitate 11 was equally as active as the untreated serum kept at the same (5°C.) temperature. There was approximately a 15 per cent reduction in the agglutinating activity in 8 and 25 per cent in Tube 4.

After 11 months Tubes 3 and 12 were similarly tested. Precipitate 12 was completely soluble and had a titer equal to that of the untreated serum. The precipitate in Tube 3 was incompletely soluble and there was a loss of approximately 50 per cent in agglutinating activity.

Thus there was complete retention of solubility and agglutinating activity of the dried serum after 11 months when kept at 5°C. At higher temperatures there was a loss in solubility and agglutinating activity. It should be noted that the conditions to which these dried preparations were subjected were somewhat extreme. No effort was made to protect the materials from the air. Possibly somewhat less loss in solubility would occur if the material were placed in partially evacuated air-tight containers.

The method would seem to offer distinct possibilities as a method for preservation of immune sera for routine laboratory tests and possibly even therapeutic sera.

In preliminary tests upon hemolytic antisera and antitoxic sera there has resulted no loss in solubility or of antibody activity as a result of preparation of the dried material as listed above.

#### *Preparation of Immune Sera in a Dry State*

As stated above we have applied the findings reported for the preparation of antisera in a dry state. The method which has been found most applicable is as follows:

To 10 or more volumes of acetone add slowly with shaking 1 volume of serum. Collect the precipitate on a filter, wash once with acetone followed by three washings with anhydrous ether, the precipitated mass being stirred with a wooden spatula after each ether addition. Approximately 5 volumes of ether to each original volume of serum are required for each washing. The final white mass is spread out on the filter paper and placed in the 37°C. incubator for about 1 hour. The resulting dry mass is readily pulverized with the wooden spatula to an extremely light, white, fluffy powder. This powder (due to slow wetting) is slowly though completely soluble in distilled water or saline (0.85 per cent NaCl). If the acetone washing is replaced by absolute ethyl alcohol the final product is a little more quickly dissolved.

Absolute ethyl alcohol may be substituted for acetone as precipitating agent in the above procedure. Also 95 per cent alcohol may be used but 19 volumes of alcohol to one of serum are necessary so the final alcohol concentration does not fall below 90 per cent.

It is essential to use anhydrous ether for washing. Washing with either U.S.P.

or anesthesia ether produces a final product which is granular in nature and slightly brown in color. It is, however, completely soluble.

The results reported in Fig. 2 and Table I indicate the necessity of working at temperatures below 35°C. and of carrying out the filtration and washing with minimal loss of time. We have encountered no loss in solubility or in antibody activity when the dried material is thus prepared at room temperature (20–25°C.) and the duration of exposure does not exceed 1 hour.

#### DISCUSSION

The phenomenon of precipitation of serum proteins in high concentration of organic solvents at room temperature has much practical and theoretical significance. From a practical standpoint it provided a rapid and effective method of reducing immune sera to a dry powder. This simplifies preservation and indications are that it increases keeping qualities. In addition it opens up a new field of approach to the purification and concentration of therapeutic sera.

From a theoretical standpoint it is interesting that such a critical concentration exists. There is undoubtedly some relationship between this coagulation phenomenon and the greater germicidal effect of 70 per cent alcohol than 95 per cent alcohol.

The results reported above indicate that denaturation does not necessarily occur at the time of precipitation, since proteins precipitated in 95 per cent alcohol are rendered insoluble by diluting the alcohol with water to 70 per cent. Furthermore proteins precipitated at 5°C. in 70 per cent alcohol are resolvable, but if the temperature of the precipitate is allowed to rise to 25°C. without altering the alcohol concentration a large part of the precipitate becomes insoluble.

#### SUMMARY AND CONCLUSIONS

1. In concentrations of 70 to 75 per cent the organic solvents methyl, ethyl, and propyl alcohols, and acetone cause complete precipitation of serum proteins and produce maximum loss in solubility. We have referred to this concentration range as the critical concentration.
2. As the concentration of the solvents is increased from about 75 per cent precipitation continues complete but loss in solubility progresses

sively decreases until at all concentrations above about 87 per cent the precipitates formed at room temperature are completely soluble.

3. The degree of resolubility of the precipitates formed even in these high concentrations of the organic solvent decreases as the temperature is raised and as the duration of exposure is increased.

4. At 5°C. the precipitates formed in all concentrations of these organic solvents are completely resoluble. Also these solvents exert maximum precipitating effect at lower temperature.

5. Maximum precipitating effect by these organic solvents occurs at about pH 6.0 precipitation becoming progressively less as the pH value is altered either way from this point.

6. The more concentrated the serum, the greater the proportion of protein present that will be precipitated by any given concentrations of organic solvent.

7. A method for preparing dry immune sera has been given. Such dried sera have been extracted with a number of organic compounds without loss in solubility or antibody activity.

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# THE EFFECT OF TEMPERATURE ON THE TITRATION CURVE OF CASEIN

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## I

### INTRODUCTION

Hoffman and Gortner (1) recently investigated the acid and base-binding properties of various proteins, among them casein. In respect to the influence of temperature, their conclusion was "... that at 35°C. much less acid or alkali is bound by the protein than at 25°C., while considerably more is bound at 15°C. than at 25°C."

Contemporary development in the theory of ionization of ampholytes points to the peculiar significance of the effect of temperature. We therefore undertook a recalculation of some of Hoffman and Gortner's data to bring their findings into conformity with recent advances in the theory of ampholytes. We believe that our investigation makes apparent certain phenomena which are not visible in the conclusions of Hoffman and Gortner. Furthermore it seems to us that neither the experimental error, nor certain suppositions made in the recalculations in any way vitiate the qualitative side of the conclusions. The quantitative side, on an absolute scale, is much less certain. We must remember the disheartening fact that there exist many casein preparations and probably two or more caseins, and also that the measurements of Hoffman and Gortner were made in a cell involving a liquid junction.

The recalculation itself is somewhat involved and may be subdivided into the following steps: first, the calculation of the E.M.F. of the half cell used by Hoffman and Gortner (1) at several temperatures; secondly, the calculation of the pH of casein solutions with acid or base at various temperatures, using these values, and finally the cal-



culution of the acid or base bound by casein at various temperatures. These calculations are described below in detail.

## II

### *The Calculation of the E.M.F. of the 1.0 N KCl Calomel Half Cell at Various Temperatures*

In their measurements Hoffman and Gortner (1) used the cell:



They calculated the pH with the help of Schmidt and Hoagland's tables (2) and the amount of acid or base bound by the proteins, by a method described in detail in their communication. They believed that "... no temperature correction was necessary in this method of calculation as the hydrogen ion concentration was used only to determine the normality of the equilibrium solution" (3).

The hydrogen ion activity of hydrochloric acid is related to the E.M.F.'s observed and to the reference electrode by the familiar expression:

$$\text{pH} = \text{p}\gamma_{\text{H}^+} + \text{pHCl} = \frac{\text{E.M.F.}_{\text{observed}} - \text{E.M.F.}_{\text{calomel}}}{0.0001984 T} \quad (1)$$

in which  $\text{p}\gamma_{\text{H}^+}$  is the negative logarithm of the hydrogen ion activity coefficient and  $\text{pHCl}$  is the negative logarithm of the normality of hydrochloric acid.

In their investigation Hoffman and Gortner (4: Table XX) carried numerous measurements with such cells at 15°, 25°, and 35°C., in which both the hydrochloric acid solution and the reference electrode were kept at the same temperature.<sup>1</sup>

It is apparent from Relation I that if the E.M.F. of the calomel half cell is unknown, as it is safe to assume in our case, it could be calculated, provided the values of  $\text{p}\gamma_{\text{H}^+}$  were known, since all other values are experimentally given.

There are several determinations of  $\gamma_{\text{H}^+}$  of hydrochloric acid available. In this calculation we accepted the  $\gamma_{\text{H}^+}$  as given by Lewis and Randall (5). A smooth curve was drawn through these values. The  $\gamma$  for 0.1 N HCl determined by Scatchard as given by Clark (6) agrees very well with the values given by Lewis and Randall. From this

<sup>1</sup> Private communication by Professor Gortner to one of the writers.

curve the values recorded in Table I, Column (2) have been interpolated.

Whether the activity coefficient of individual ions can be actually determined, is a somewhat debatable question. But, as we shall see presently, in the comparison of the properties of casein at various temperatures  $\gamma_{H^+}$  is involved as a constant. Therefore its exact value is immaterial. This conclusion follows from the following

TABLE I

*The E. M. F. of 1.0 N KCl Calomel Half Cell at Various Temperatures*

HCl normality	$\gamma_{H^+}$	E.M.F. of the half cell at:		
		15°	25°	35°
(1)	(2)	(3)	(4)	(5)
0.003	0.965	0.2860	(0.2755)	(0.2705)
0.006	0.945	0.2875	0.2810	0.2760
0.009	0.935	0.2875	0.2815	0.2765
0.012	0.925	0.2870	0.2815	0.2770
0.018	0.910	0.2855	0.2815	0.2775
0.024	0.900	0.2850	0.2805	0.2770
0.030	0.890	0.2850	0.2810	0.2780
0.045	0.875	0.2855	0.2810	0.2770
0.060	0.860	0.2845	0.2805	0.2775
0.075	0.855	0.2835	0.2790	0.2750
0.090	0.845	0.2840	0.2790	0.2755
0.105	0.835	0.2845	0.2790	0.2760
0.120	0.835	0.2850	0.2780	0.2755
0.150	0.825	0.2840	0.2780	0.2745
Average accepted.....		0.2855	0.2800	0.2765

considerations. In dilute solutions (to about 0.15 N) the activity coefficient of hydrochloric acid is practically independent of temperature (7, 8). It is therefore possible to use the same value of  $\gamma_{H^+}$  at different temperatures provided sufficiently dilute hydrochloric acid solutions are used.

We are now in a position to calculate the E.M.F. of the half cell Sat. KCl | 1 N KCl, HgCl | Hg from the data of Hoffman and Gortner (4: Table XX) with the help of Equation I. This has been done in Table I where the E.M.F.'s of this reference electrode are calculated

at three different temperatures and at normalities of hydrochloric acid ranging from 0.003 to 0.150.

The values obtained are far from perfect. They display variations greater than such measurements should warrant. But this fluctuation cannot be remedied by a selection of another set of  $\gamma$  and is probably inherent in the measurements themselves. They are however sufficiently accurate for our purpose.

The casein solutions in which we are interested were measured by Hoffman and Gortner at  $22 \pm 1^\circ$  and  $35^\circ\text{C}$ . The value of the reference electrode at  $22^\circ$  was interpolated from a smooth curve down through values obtained in Table I.

### III

#### *The Amount of Acid or Base Bound by Casein at Various Temperatures*

As a next step we recalculated the hydrogen ion activities of casein solutions in equilibrium with sodium hydroxide and hydrochloric acid. This was done with the help of Equation I, the E.M.F.'s of the calomel half cell arrived at in the preceding section, and the E.M.F.'s observed by Hoffman and Gortner (1: Tables LXXX, LXXXIV, LXXXVIII, and XCII). The data cover the titration of casein with moderate amounts of HCl and NaOH at  $22^\circ$  and  $35^\circ\text{C}$ .

The pH values of the system casein-sodium hydroxide were converted into pOH terms by subtracting the pH values from 14.100 at  $22^\circ$  and from 13.670 at  $35^\circ$ .

The results of these calculations are found in Figs. 1 and 2, except for larger concentrations of HCl and NaOH. The data in this case were corrected for the free NaOH or HCl in the following manner: Cohn (9) suggests that the amount of NaOH bound by the protein might be obtained from the following considerations:

$$\text{pH} + \text{pOH} = \text{p}K_w \quad (\text{II})$$

$$\text{pOH} + \text{p}\gamma_{\text{OH}} = \text{pNaOH} \quad (\text{III})$$

$$\text{NaOH added} - \text{NaOH free} = \text{NaOH bound} \quad (\text{IV})$$

Experimentally we know the pH; using the  $\text{p}K_w$  given above we can find the pOH. Taking the values given by Cohn (9) for the activity coefficients of OH, we can obtain pNaOH and NaOH free. The

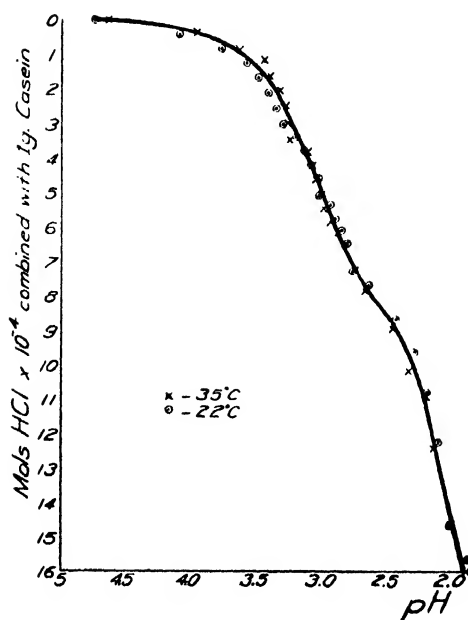


FIG. 1. The titration curve of casein with hydrochloric acid at 22° and 35°C.

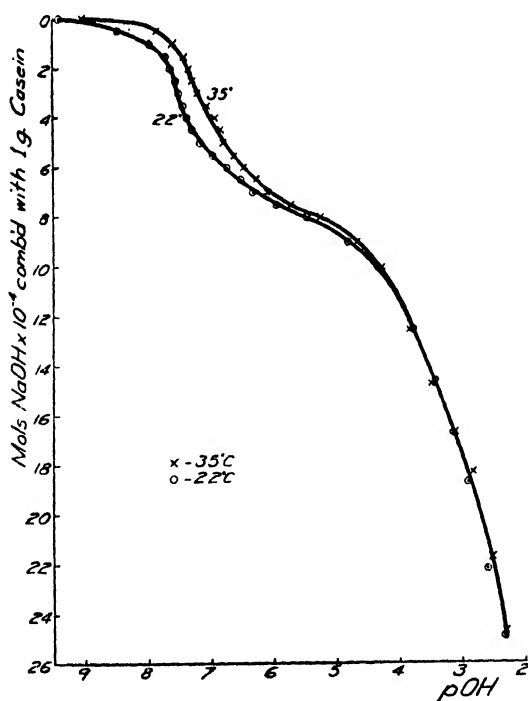


FIG. 2. The titration curves of casein with sodium hydroxide at 22° and 35°C.

latter is the antilog of pNaOH. Subtracting the free NaOH from the total NaOH added, we obtain the value for the NaOH bound by the protein.

A similar calculation was carried out for hydrochloric acid using the activity coefficients given in Table I.

The correction for the free NaOH is necessary only through a small region of the titration curve with NaOH. In the case of HCl it is significant almost throughout the titration curve.

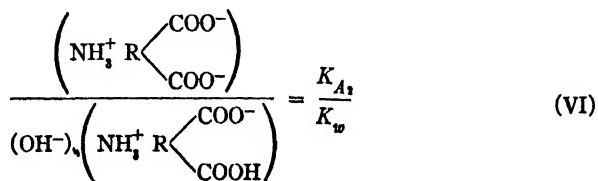
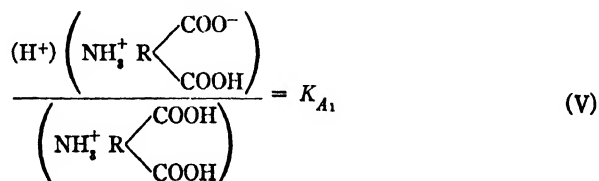
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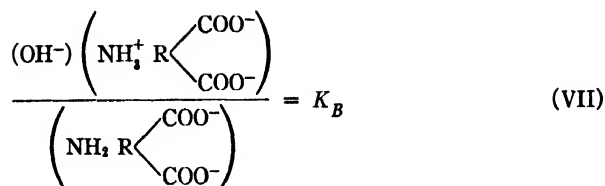
#### DISCUSSION

In Figs. 1 and 2 are reproduced the results of these calculations. A superficial examination of these data convinces us that we are dealing with two distinct phenomena in the titration of casein with sodium hydroxide. In terms of the negative logarithm of the hydroxyl ion activity (pOH) the effect of the temperature is considerable beginning from the isoelectric point to about pOH 6 (pH 8), but in a more alkaline region the two curves practically coincide.

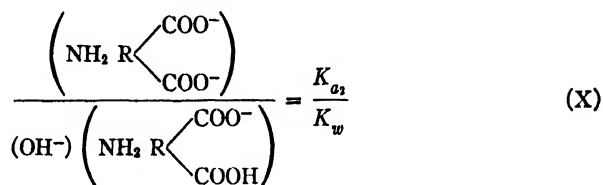
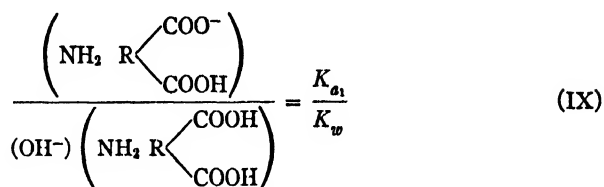
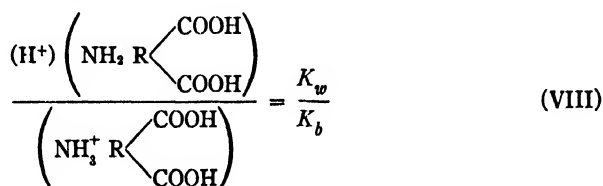
Similarly, the acid side of the titration curve in terms of pH (Fig. 1) is practically identical at 22° and 35°C. There is a slight discrepancy between the two curves between pH 4 and 3.2, but casein in combination with acid is slow in coming to equilibrium and we are rather inclined to attribute this discrepancy to an experimental error.

In 1923 Bjerrum (10, 11) suggested that a dicarboxylic amino acid should dissociate in the following way:





The corresponding constants according to the classical dissociation theory are (in the same order):



At first the whole problem seems to be one of notation, but on more careful examination we may note the following important differences:

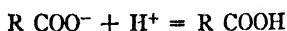
1. According to the classical theory the dissociation of an amino acid in acid solution, in terms of  $\text{H}^+$ , involves  $K_w$  (VIII), in the case of the new theory (V) it does not.

2. In the case of the second classical constant,  $K_{a2}$  (X), in terms of  $\text{OH}^-$ , the situation is the same as in 1.

3. In the case of the first classical dissociation constant,  $K_{a1}$  (IX), as far as  $K_w$  is concerned, there is no difference between the old and new theory.

Casein in many ways resembles a multiple monoamino-dicarboxylic acid (12, 11). The important difference however is that these reacting groups are numerous since the molecular weight of casein is in the neighborhood of 200,000.

In regard to the effect of temperature on the types of ionization described in Equations V to X we know that the  $K_w$  is considerably influenced by the temperature ( $\Delta H$  equals about 14,000 calories), while the ionization described by other constants is, or may be, little affected. For instance the reaction:



involves only from  $-1,300$  to  $+1,800$  calories (11). To be more specific one must expect that equilibrium (Equations V to X) involving the  $K_w$  must be very much influenced by temperature, while those which do not include the  $K_w$  are likely to be little affected.

In applying these criteria to the experimental findings (Figs. 1 and 2) we at once see that the type of ionization suggested by Bjerrum fits the experimental facts in a far better way than the classical one:

1. On the acid side of the titration curve of casein (Fig. 1) the classical theory predicts (VIII) a large temperature coefficient, Bjerrum's (V) a small one. The experimental findings indicate a small temperature coefficient.

2. On the alkaline side of the titration curve of casein (more alkaline than about pOH 6, Fig. 2) the classical theory (X) suggests a large temperature coefficient, while the theory of Bjerrum (VIII) a small one. The experimental facts again favor the Bjerrum theory.

3. In the range from the isoelectric point to about pOH 6 both theories predict a large temperature coefficient (Equations VI and IX). The experimental facts are in perfect agreement on this point.

On the whole, we must therefore conclude that the type of ionization suggested by Bjerrum adequately describes the effect of temperature on the titration curve of casein with acid or base. The study of the effect of temperature on the titration curves of proteins may thus become a useful tool in identifying the various types of ionization involved.

## V

### SUMMARY

The influence of temperature on the titration curve of casein may be accounted for by the Bjerrum theory of ionization of ampholytes.

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# CRYSTALLINE TRYPSIN

## I. ISOLATION AND TESTS OF PURITY

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It has been known since the time of Corvissart and Kühne that pancreatic juice possesses the property of digesting proteins. Kühne assumed that this property was due to the presence in the juice of an unorganized ferment or enzyme which he called trypsin. Subsequent work by Fisher and Abderhalden, Cohnheim, Bayliss, Vernon, Schaffer and Terroine, Abderhalden, Willstätter, and Waldschmidt-Leitz and their collaborators has added greatly to our knowledge of the enzymatic properties of pancreatic juice (1). The kinetics of these reactions have also been partially worked out. According to Waldschmidt-Leitz the activated pancreatic juice contains at least five proteolytic enzymes—trypsinogen, trypsin-kinase, carboxy-peptidase and amino-peptidase, which hydrolyze polypeptides, and erepsin. There is no positive evidence<sup>1</sup> as yet that any of these enzymes has ever been obtained in pure form or separated completely from each other and it is probable that the separation can be carried still further, as already indicated by Abderhalden (3). The enzyme described in this paper differs essentially from any previously described. In the meantime the existence of these separate enzymes has been assumed purely from the behavior of certain solutions, as Linderström-Lang has pointed out (4).

In contrast to the marked advances in knowledge of the properties

<sup>1</sup> In one sense this will always be the case since it is not possible to furnish positive proof of the purity of a substance but only negative proof consisting of the fact that no evidence of mixtures can be found under conditions which would be expected to show such evidence. For a discussion of the difficulty of proving the existence of, or defining a "pure substance" see Lunn and Senior (2).

of the enzymes of the pancreatic juices, as shown by their catalytic effect in various reactions, little or no knowledge has been gained as to the chemical nature of these enzymes. The early workers, Kühne, Mays, Hammarsten, and Michaelis, considered them to be associated with the nucleic acid fraction; but Levene (5) showed that they were not nucleic acids themselves since hydrolysis of the nucleic acids did not destroy the activity. Willstätter and his collaborators (6) concluded that the enzymes belong to an unknown class of chemical compounds associated with proteins or other substances of high molecular weight and a somewhat similar point of view is expressed by Fodor (7). This conclusion, however, rests only on the purely negative evidence that no pure substance, *i.e.* one having constant properties, has been obtained which showed enzymatic activity. The known chemical properties of enzymes, on the other hand, such as the temperature coefficient of inactivation, effect of acid and alkali on stability, and the reactivation sometimes observed, as well as the ease with which they are adsorbed on colloidal particles, are those of proteins. In addition, crystalline proteins of constant composition and activity have been isolated by Sumner (8) in the case of urease, by Northrop (9) in the case of pepsin, and by Caldwell, Booher, and Sherman (10) in the case of amylase. The existing positive evidence, therefore, indicates the protein nature of enzymes and the conditions and methods used in the present work were those known to be favorable for the isolation of proteins; *i.e.*, concentrated solutions in concentrated neutral salt and low temperatures. These methods have led to the isolation of a crystalline protein having constant physical and chemical properties, including constant proteolytic activity. This enzyme attacks only proteins, and pepsin-peptone, so far as we have determined. Since the protease of pancreas has always been called trypsin the present enzyme will be referred to as trypsin. It differs markedly from the trypsin-kinase of Waldschmidt-Leitz in that it does not carry the hydrolysis of proteins nearly so far as does trypsin-kinase.

#### *Methods of Determining Activity*

The activity of the various preparations was determined by the change in viscosity of gelatin and casein, the increase in formol titra-

tion of gelatin and casein, and the formation of non-protein nitrogen in casein solutions, etc. The following definitions and abbreviations are used in the paper.

$[T. U.]_{mg. N}^{4g V}$ —per cent change in viscosity per minute per mg. trypsin nitrogen contained in 5.2 ml. 2.5 per cent gelatin, pH 4.0.

$[T. U.]_{mg. N}^{Cas. F}$ —milliequivalents carboxyl groups liberated per minute, per mg. trypsin nitrogen contained in 6.0 ml. 4 per cent  $\left\{ \begin{array}{l} \text{casein} \\ \text{gelatin, pH 7.6.} \end{array} \right.$

$[T. U.]_{mg. N}^{Cas. S}$ —milliequivalents nitrogen soluble in 5 per cent trichloroacetic acid formed per minute per mg. trypsin nitrogen contained in 6.0 ml. 4 per cent casein, pH 7.6

$[T. U.]_{mg. N}^{Ren.}$ —per cent increase in viscosity per minute per mg. trypsin nitrogen contained in 5.2 ml. of standard milk solution.

$[T. U.]_{mg. N}^{Clot.}$ —ml. magnesium sulfate plasma clotted by 1 mg. trypsin nitrogen in 18 hours, 6°C.

$[\alpha]_{mg. P. N.}^D$ —degrees optical rotation in a 1 dm. tube of a solution in  $\frac{1}{4}$  saturated ammonium sulfate, M/10 pH 4.0 acetate, containing 1 mg. protein nitrogen per ml.; sodium *D* line, at 20°C.

The methods used are described in the experimental part of the paper (11).

The determinations were confined in all cases except the rennet action, to the initial slope of the curves in which region the specific activity obtained is independent of the concentration of enzyme used.

### *Preliminary Method of Fractionation*

The first attempts at purification of the enzyme were similar to the experiments of Michaelis and Davidsohn (12) and consisted in a study of the precipitate obtained from crude trypsin extracts at about pH 3.0. The crude material used was Fairchild's trypsin which is prepared from beef pancreas.<sup>2</sup> If an aqueous extract of the dry powder is titrated to about pH 3.0 with acid a precipitate forms, as Michaelis found. Most of the activity is found in this precipitate. Upon repeated solution and reprecipitation much of the activity is lost and the specific activity of the precipitate becomes less and less. A number of other methods of fractionation were tried but it was found that

<sup>2</sup> The writers are indebted to Mr. Benjamin Fairchild of Fairchild Bros. and Foster for this information.

the precipitate obtained with strong ammonium sulfate was the only one which could be dissolved and reprecipitated indefinitely without loss of activity either as a whole or in regard to the precipitate. Systematic fractionation of an extract of the commercial preparation in  $\frac{1}{4}$  saturated ammonium sulfate was then undertaken with various concentrations of ammonium sulfate. As the ammonium sulfate concentration is increased a series of precipitates is obtained which become more and more active. The most active precipitate appeared at about 0.6 saturated ammonium sulfate and further fractionation of this precipitate did not change its activity appreciably. The precipitate was a protein and gave some indications of crystallizing so that a large number of experiments were done in an attempt to crystallize it. It was found eventually that crystals could be obtained by adding saturated ammonium sulfate very cautiously to a 5 per cent solution of the precipitate (1 part filter cake dissolved in five times its weight of solution) in  $\frac{1}{4}$  saturated ammonium sulfate made up in M/10 acetate buffer, pH 4.0, temperature 25–30°C. The first precipitate which appears under these conditions is usually amorphous. If this is filtered off and the filtrate allowed to stand at 25–30°C., small crystals of cubic form begin to appear in the solution and increase rapidly in amount. Good crystals are obtained only with slow crystallization. Otherwise the crystals are not well formed and usually appear spherical. The process is favored by stirring. The crystallization may be hastened by continued addition of ammonium sulfate (with stirring) and eventually practically all the protein may be obtained in crystalline form. This is the method of preparation already reported (13). Material obtained in this way is about ten times as active as the original commercial product on a total dry weight basis and about three times as active per milligram soluble protein nitrogen.

Repeated crystallization does not change the specific activity to any extent but it was noted that the first small amount of precipitate formed was always slightly less active than the succeeding crops of crystals. The results of such an experiment are shown in Table I.

It was very difficult to decide whether this result was due to actual fractionation or to loss in activity during the experiment. In order to test the purity of the preparation in another way a series of solubility experiments was done in  $\frac{3}{4}$  saturated magnesium sulfate. The crys-

talline precipitate was stirred for 10 to 15 minutes with a mixture made up of 75 ml. saturated magnesium sulfate and 25 ml.  $M/10$  pH 4.0 acetate. The suspension was then filtered and the precipitate again

TABLE I

100 gm. poorly crystalline cake dissolved in 600 ml.  $\frac{1}{2}$  saturated ammonium sulfate in  $M/10$  acetate buffer, pH 4.0. Saturated ammonium sulfate added with stirring to faint turbidity. More saturated ammonium sulfate run in very slowly. Crystalline precipitate formed and filtered off from time to time. Saturated ammonium sulfate added to filtrate until no further crystals are obtained. The precipitates were then combined, dissolved in 6 volumes  $\frac{1}{2}$  saturated ammonium sulfate, pH 4.0 and the crystallization of Fraction b repeated four times. Samples of each fraction were analyzed for protein nitrogen, optical activity, and proteolytic activity by gelatin viscosity method.

Crystallization No.	Weight of cake	Fraction	Precipitate	[T. U.] $\frac{4gV}{mg. N}$	$[\alpha]_D^{25}$ $\frac{D}{mg. N}$
1	100	1 a	11	40	0.33
		1 b	17	55	
		1 c	15	57	
		1 d	11	57	
		Mother liquor		42	
2 (1a + 1b + 1c + 1d)	39	2 a	1.3	42	0.33
		2 b	26.5	47	
		Mother liquor		36	
3	26	3 a	2.5	43	0.37
		3 b	11.5	47	
		Mother liquor		50	
4	11	4 a	1		0.39
		4 b	7.5	45	
		Mother liquor		40	
5	7	5 a	0.5	42	0.36
		5 b	3.6	45	
		Mother liquor			

stirred with the same solvent. This was continued until the precipitate had nearly all gone into solution and the filtrates and residue then analyzed for nitrogen and activity. The results of the experiments are shown in Table II.

The solubility decreases rapidly with successive extractions while the specific activity of the protein in solution also decreases to some extent. The final residue, however, has about the same activity as the original material. These results show definitely that the crystalline material prepared by this method is not a pure protein since the solubility depends on the quantity of precipitate. It is similar to the type of result found by Sørensen (22) with other proteins and indicates that the substance is probably a solid solution. There was some loss in activity during this experiment but hardly sufficient to account for the observed results.

TABLE II

*Solubility in Magnesium Sulfate, pH 4.0 Acetate =*  
*{ 75 Ml. Saturated Magnesium Sulfate*  
*25 Ml. M/10 pH 4.0 Acetate*

20 gm. crystalline filter cake stirred for 10-15 minutes with 75 ml. solvent. Filtered and filtrate analyzed for total nitrogen and activity. Extraction repeated eight times.

Extract No.....	1	3	5	7	9	Residue from 9th extract
N/ml.....	1.92	0.94	0.80	0.49	0.29	
[T. U.] <sup>4gV</sup> mg. P. N. ....	60	36	54	40	47	60

### *Effect of Heating in Acid Solution*

It was noted when a solution of the material was boiled in dilute hydrochloric acid and then cooled that a form of protein appeared which precipitated on the addition of magnesium sulfate although there was not very much loss in activity. This remarkable stability in dilute acid is a characteristic property of trypsin and has been noted by Mellanby and Wooley (14). It was thought at first that the precipitate formed on adding a solution, which had been heated and then cooled, to salt solutions was probably denatured trypsin protein and an experiment was made in order to determine whether the loss in activity was proportional to the quantity of this denatured protein formed. A solution of the material in N/10 hydrochloric acid was heated to 95°C. and the total activity of the solution

determined as well as the quantity of denatured protein present. The results of an experiment of this kind are shown in Table III.

The table shows that the total protein nitrogen remains constant while the activity per milliliter of solution decreases. The specific activity per milligram total protein nitrogen therefore decreases with time. However, if instead of total protein nitrogen the concentration of protein which does not precipitate with magnesium sulfate is considered, the experiment shows that the specific activity, referred to this soluble protein, increases nearly 100 per cent in the first few min-

TABLE III

*Loss in Activity and Formation of Denatured Protein in Dilute Acid Solution at 95°C.*

2 gm. of crystalline filter cake dissolved in 50 ml. of *M*/10 hydrochloric acid and kept at 95°C. 1 ml. samples taken and added to 4 ml. *M*/10 pH 4.0 acetate buffer and this solution analyzed for protein nitrogen and total activity. Another set of 1 ml. samples taken, cooled, and added to 4 ml. cold *M*/10 magnesium sulfate in 0.5 normal sulfuric acid. The suspension centrifuged and supernatant liquid analyzed for protein nitrogen and activity.

Time	Analysis from samples in acetate			Analysis of filtrate from MgSO <sub>4</sub> samples		
	Protein N	[T. U.] <sup>4gV</sup> <sub>ml.</sub>	[T. U.] <sup>4gV</sup> <sub>mg. P. N.</sub>	Protein N	[T. U.] <sup>4gV</sup> <sub>ml.</sub>	[T. U.] <sup>4gV</sup> <sub>mg. P. N.</sub>
<i>hrs.</i>	<i>mg.</i>			<i>mg.</i>		
0	1.6	100	62	1.6	100	62
0.1	1.57	80	51	0.58	63	110
0.2	1.5	64	43	0.56	54	97
0.4	1.7	43	25	0.52	27	52
0.8	1.7	18.5	11	0.45	7.4	16

utes, remains nearly constant for a while, and then decreases. The experiment shows conclusively that the material is not a pure substance but contains a protein which is denatured by heat and which has little or no activity, and another protein of high activity which either is not denatured by heat or which reverts to the native condition on cooling.<sup>3</sup> This behavior is so unusual that a detailed study of

<sup>3</sup> This result is very similar to that obtained recently by Waldschmidt-Leitz and Steigerwaldt (15) in following the digestion of urease with trypsin. It proves only that the material is not a pure protein but does not prove that no active protein is present.



the reaction was made and is reported in another paper (16). It turns out that the active protein is denatured when heated but reverts to the native condition very rapidly on cooling. At the same time the activity returns.

The experiment just described, however, also indicates a very efficient method of further fractionation. A study of the fractionation was therefore undertaken again. The raw material was either an aqueous extract of Fairchild's or other commercial trypsin, or pancreatic juice obtained from frozen pancreas. The frozen mass was sliced, spread on racks and allowed to thaw, and the liquid which drained out collected.

#### *Final Method of Isolation*

The method of fractionation finally worked out consists essentially of a preliminary precipitation of juice obtained in this way with strong acid which removes most of the inert proteins, as has previously been reported by Fodor and by Schönfeld-Reiner (17). The filtrate from this precipitate is then fractionated with ammonium sulfate and dissolved in dilute acid, heated to 80°C., and again fractionated with ammonium sulfate. By this method a protein was obtained which had about twice the specific activity of the original crystalline material but which crystallized under the same conditions and in a similar crystalline form (*cf.* Fig. 1). Up to the present it has not been found possible to increase the specific activity any further. Whenever any of the protein is destroyed or removed there is a corresponding loss in activity. The details of the preparation are shown in Table IV. In this experiment the heating and fractionation were repeated four times. Ordinarily the fraction obtained after the first heating (No. 5 in the table) is used as the final material. This fraction contains nearly one-half of the original proteolytic activity. Most of the loss occurs when the solution is heated. This is due to the fact that there are other proteins present in the solution, since, as is described in the paper on heat inactivation (16), the purified material may be heated and cooled again indefinitely without any loss in activity. The specific activity is about six times that of the original crude extract on the basis of protein nitrogen and considerably more than six times as active on the basis of total dry weight.

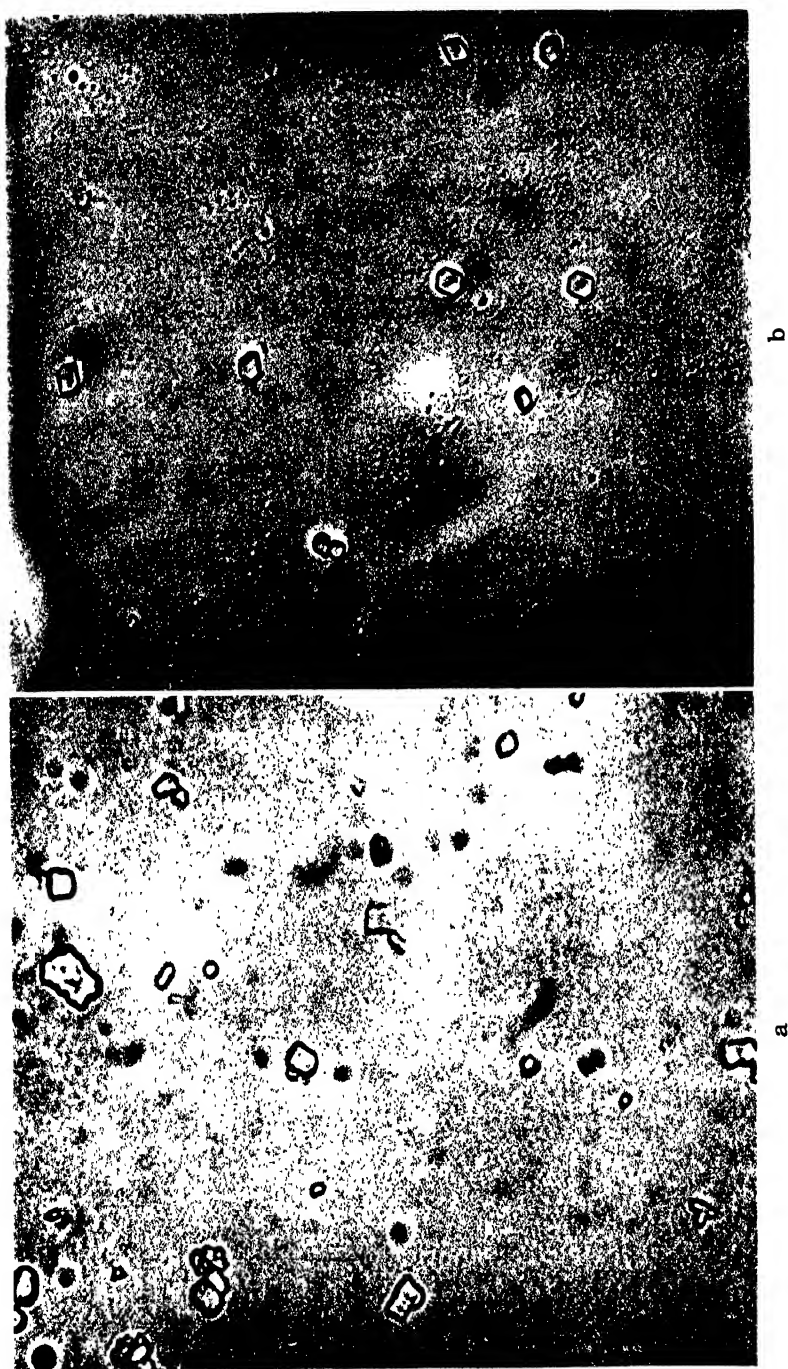


FIG. 1. *a* and *b*. (*a*) Trypsin crystals immediately after crystallization. (*b*) Trypsin crystals after standing in contact with the mother liquor for 1 or 2 months.

TABLE IV  
*Preparation of Crystalline Trypsin*

	Fraction No.	Weight gm.	[T. U.] <sup>4gV</sup> mg. P. N.	Total [T. U.] <sup>4gV</sup>
150 kg. frozen pancreas cut in slices and allowed to thaw overnight at about 5°C. and the expressed fluid collected	1	12,000	18	$12 \times 10^5$
Diluted with 1 volume water and 22 ml. concentrated hydrochloric acid added per liter (final concentration HCl 0.25 molar). Solution filtered in cold room through fluted paper (S. and S. No. 1450 1/2). Filtrate brought to 0.4 saturated ammonium sulfate by the addition of solid ammonium sulfate (250 gm. per liter) and refiltered through fluted paper. Filtrate brought to 0.7 saturated ammonium sulfate (250 gm. ammonium sulfate per liter) and filtered with suction. Precipitate	3	520	37	$9 \times 10^5$
500 gm. Precipitate 3 dissolved in 12 liters N/20 hydrochloric acid, heated* rapidly to 90°C. and cooled to 20°C., brought to 0.4 saturated ammonium sulfate, and filtered. Filtrate brought to 0.7 saturated ammonium sulfate and filtered with suction. Precipitate	5	120	95	$5 \times 10^5$
This completes the usual method of preparation. The succeeding fractionations are reported to show that no further change in activity occurs				
Precipitate 5 dissolved in 25 volumes M/20 pH 4 acetate buffer, heated rapidly to 90°C., cooled to 20°C., brought to 0.4 saturated ammonium sulfate, and filtered. Brought to 0.7 saturated ammonium sulfate and filtered with suction. Precipitate	7	70	100	$4 \times 10^5$
No. 7 dissolved in 3 volumes $\frac{1}{4}$ saturated ammonium sulfate pH 4 brought to 0.4 saturated ammonium sulfate, filtered. Filtrate brought to 0.7 saturated ammonium sulfate, filtered with suction. Precipitate	8	50	110	$3 \times 10^5$

TABLE IV—*Concluded*

	Fraction No.	Weight <i>gm.</i>	[T. U.] <sup>4gV</sup> mg. P. N.	Total [T. U.] <sup>4gV</sup>
No. 8 dissolved in 25 volumes N/20 hydrochloric acid, brought to 0.4 saturated ammonium sulfate, and filtered. Filtrate brought to 0.7 ammonium sulfate, filtered with suction. Precipitate	9	35	105	$2 \times 10^6$
No. 9 dissolved in 3 volumes $\frac{1}{2}$ saturated ammonium sulfate, brought to 0.4 saturated ammonium sulfate by the addition of saturated ammonium sulfate, clear solution. Saturated ammonium sulfate added slowly until slight turbidity. Solution filtered, filtrate inoculated with crystals, and saturated ammonium sulfate added very slowly. Crystalline precipitate forms. Precipitate	10	20	110	$1 \times 10^6$

\* The heating and cooling of large quantities of solution may be most conveniently done by running the solution through a glass coil immersed in boiling water and then through a coil in cold water. The rate of flow is regulated so that the solution leaves the hot coil at a temperature of 80–85°C. The coil used in these experiments was of 5 mm. (inside diameter) thin walled tubing, about 2 m. long.

It is possible to prepare crystalline material having the maximum activity by fractionation with ammonium sulfate alone and without heating. The process is laborious and the yield poor so that, for practical purposes, the method is not satisfactory. The material obtained in this way, however, is identical with that obtained after heating. There is no reason to suppose, therefore, that the properties of the enzyme are changed by the heating.

The various fractions were tested for tryptic activity by gelatin and casein viscosity methods at pH 4.0, by the formol titration of gelatin and casein, and by the production of soluble nitrogen from casein. They were also tested for their blood-clotting power (18), for ability to clot milk, and for amylase, lipase, and erepsin. The optical activity at pH 4.0 and the total increase in formol titration with casein and gelatin in the presence of excess enzyme were also determined. The results of these determinations are shown in Table V.

The proteolytic activity, as measured by any of the methods and

TABLE V  
*Analysis of Fractions*

Fraction No.	1	3	5	7	8	9	10	Glycerin extract of acetone dried pig pancreas	Crystalline pepsin
[T. U.] <sup>4gV</sup> mg. P. N.	18	37	100	100	110	105	110	20	13
[T. U.] <sup>Gel. F</sup> mg. P. N.	0.05	0.11	0.32	0.24	0.34	0.33	0.29		0.001
[T. U.] <sup>Cas. V</sup> mg. P. N.	160	330					870		
[T. U.] <sup>Cas. F</sup> mg. P. N.	0.053	0.084	0.19 0.13	0.15	0.16 0.12	0.23	0.18	0.04	0.20
[T. U.] <sup>Cas. S</sup> mg. P. N.	0.61	1.1	2.4 1.9	1.7	2.1	2.2	2.4	0.50	0.44
[T. U.] <sup>Clot.</sup> mg. P. N.		810		1500			1500		
[ $\alpha$ ] <sup>D</sup> mg. N (1 dm. tube) 25°C.		-0.458		-0.28	-0.29		-0.26		
Amylase—mg. P. N. for positive test, mg.	<0.001	>2		>2	>2	>2	>2		

[T. U.] <sup>Ren.</sup> mg. P. N.	1000	4000	400	300	160	100	95	280,000
Lipase— ml. n/10 1 mg. N	0.6	0.4						
Erepsin (glycyl-glycine)	+	±		—	—	—	—	
Maximum increase in formol titration— ml. n/50 NaOH per 5 ml. protein solution	>32	>15		9.1	9.0	8.9	9.0	17.0
	>25	>10		6.9	7.0	7.0	7.0	7.0

also the blood-clotting property increases rapidly up to Fraction 5 and then remains constant. The percentage increase in activity between Fraction 1 and Fraction 5 is slightly more as determined by the gelatin viscosity method than by the other methods, which may be due either to the presence of more than one enzyme, one of which is removed during the course of purification, or to the fact that the other methods of measuring the activity measure secondary reactions due to peptone-splitting enzymes while the viscosity method measures only the change in the protein. The results as a whole indicate that the final preparation has constant activity as measured by any of the preceding methods and that no change in this activity occurs during the repeated fractionations between Fraction 5 and Fraction 10. The optical activity per milligram of nitrogen also reaches a constant value. The amylase activity is expressed as the number of milligrams protein nitrogen required to give a positive test under the conditions used by Willstätter. Fraction 1 has powerful amylolytic activity but no positive test for amylase can be obtained in succeeding fractions. The lipase activity, which is expressed as ml. N/10 alkali per milligram nitrogen under the conditions described in the experimental part (11) disappears in Fraction 5 as does the ability to digest dipeptides. The rennet action decreases rapidly as fractionation proceeds but even the last fractions have a weak effect on the clotting of milk which seems to remain constant. There is no doubt that there is originally present another enzyme with a much more powerful milk-clotting power than the final trypsin and it is possible that the small amount of rennet action noted with the final fractions is due to a trace of this enzyme carried through the preparation.

The specific activity of an activated glycerin extract of dried pancreas prepared according to Willstätter's method (19) is included for comparison. It has about the same specific activity on the basis of protein nitrogen as does the pancreatic extract used as the starting point of this fractionation. The specific activity of crystalline pepsin (20) is also shown in Table V. Pepsin is less active by most of the methods but very much more active in the clotting of milk. The relative activity of the two enzymes expressed in this way cannot be considered an absolute figure since the value obtained would vary with the conditions and methods used for the determination of the activity.

*Hydrolysis of Peptides.*—Waldschmidt-Leitz (24) has separated two enzymes from pancreatic extract, carboxy-polypeptidase and aminopolypeptidase capable of splitting some peptides, while his trypsin-kinase does not attack these compounds. According to Abderhalden (25), however, trypsin-kinase solution on standing in glycerin recovers its power to hydrolyze leucyl-glycine and other dipeptides. It was of interest, therefore, to determine the activity of the trypsin fraction obtained in the present experiments with peptides. The writers are indebted to Professor Waldschmidt-Leitz for kindly supplying them with some *d*-*l*-leucyl-*l*-tyrosine, leucyl diglycine, and chlor-

TABLE VI

*Hydrolysis of Dipeptides with Various Trypsin Fractions*

20 ml. *m*/25 peptide solution pH 7.5 in *m*/10 pH 7.5 phosphate buffer, about 5 mg. trypsin protein nitrogen added, 24 hours at 35°C. 5.0 ml. titrated with *N*/50 sodium hydroxide (formol titration).

Peptide	<i>d</i> - <i>l</i> -Leucyl- <i>l</i> -tyrosine	Leucyl diglycine	Chloracetyl- <i>l</i> -tyrosine	Chloracetyl-leucine	Glycyl-alanine	Glycyl-glycine	Glycyl aspartic acid
Hydrolysis after 24 hrs. with various trypsin preparations							
Trypsin preparation No.	per cent	per cent	per cent	per cent	per cent	per cent	per cent
1.....	25	30	100	70	14	12	7.5
3.....			0				
Crystalline.....	0	0	0	0	0	0	0

acetyl-*l*-tyrosine. 1/25th molar solutions of the preparations were prepared in *m*/10 phosphate buffer and titrated to pH 7.6. 1 ml. of various trypsin fractions containing 5 mg. of protein nitrogen was then added to 20 ml. of the peptide solution and hydrolysis allowed to proceed for 24 hours at 37°C. 5 ml. samples were then titrated with *N*/50 alkali in the presence of formalin, as usual. The method of titration is accurate to about 0.1 ml. *N*/50 alkali, which corresponds to a hydrolysis of about 1 per cent. The results of the experiment, expressed as per cent of the total hydrolysis are shown in Table VI. The original extract is quite active, especially with chloracetyl-*l*-tyrosine and therefore contains considerable carboxyl-polypeptidase.



The activity with respect to chloracetyl-*L*-tyrosine is lost at the first step in the fractionation; *i.e.*, treatment with strong acid. None of the succeeding fractions showed any activity. The crystalline material possessed no measurable activity with any of the peptides and in this respect agrees with results obtained with Waldschmidt-Leitz's trypsin-kinase.

The trypsin solutions used were in 75 per cent glycerin and had been stored in the ice chest for 4 or 5 months so that there is no recovery of activity under these conditions, as reported by Abderhalden for trypsin-kinase. In order to confirm this result a solution of trypsin was made up in 75 per cent glycerin, titrated to pH 7.5, and kept at 37°C. for 10 days. These conditions are described by Abderhalden (25) as favorable for the recovery of the activity. The solution of crystalline trypsin, however, remained inactive toward the peptides under these conditions so that in this respect it differs from the trypsin-kinase. The addition of glycine to the digestion mixture did not result in any activity (25).

### *Extent of Hydrolysis*

The most striking difference between the various fractions is the extent to which the hydrolysis of casein and gelatin is carried when measured by the formol titration. The crude material causes a maximum increase in formol titration of more than 32 ml. N/50 sodium hydroxide per 5 ml. 5 per cent casein, and of more than 25 ml. per 5 ml. 5 per cent gelatin. The purified fractions only increase the formol titration of casein by 9 ml. M/50 and of gelatin by 7.0 ml. (*cf.* Paper II of this series).

### *Analysis*

The results of ultimate analysis of the protein crystals are shown in Table VII. They are similar to those usually obtained for albumin. The protein contains no phosphorus.

### *Specific Activity of Different Preparations*

About twenty different lots of crystalline trypsin have been prepared during the course of this work from different raw materials. The specific activity of most of these preparations together with that

TABLE VII

*Elementary Analysis Crystalline Trypsin*

Crystalline trypsin dissolved in N/10,000 hydrochloric acid and dialyzed against N/10,000 hydrochloric acid until free of sulfate. Poured into 10 volumes acetone at 0°, filtered. Dried to constant weight *in vacuo* at 60°C.

Element	Per cent	
	1	2
C.....	50.0	49.9
H.....	7.2	7.1
N.....	14.8	15.0
Cl.....	2.86	2.9
S.....	1.10	1.10
P.....	0.00	0.00
Ash.....	1.2	1.1

TABLE VIII

*Activity of Various Preparations*

Preparation	Raw material	Specific activity per mg. protein nitrogen by gelatin viscosity. [T. U.] <sup>4gV</sup> mg. P. N.	
		Raw material	Crystalline trypsin
<i>1931</i>			
June 17	Fairchild (Beef pancreas)	28	106
Nov. 4	Röhm and Haas, "Raw Degomma S" (pig)	7	106
" 13	Frozen beef pancreas juice	13	108, 104
" 17	" " " "	12	110, 94
" 27	" " " "	7	55
Dec. 7	" " " "	16	106, 110
<i>1932</i>			
Feb. 11	" " " "	19	85
May 18	Same material as Feb. 11 after 3 mos. in ice box		120
Mar. 14	Frozen beef pancreas juice		102
Apr. 15	" " " "		50
May 10	Fresh beef pancreas extracted 2 days at 20°C. with 1 volume water + 1 per cent cresol	8.7	62
" 13	Fairchild	25	108

of the crude material (Fraction 1) is shown in Table VIII. With the exception of four preparations, the specific activity was in each case 110 per milligram nitrogen, within the experimental error. The preparations which gave low results behaved abnormally in that heating in acid caused little or no increase in activity. It could easily be shown by repeated fractionation with ammonium sulfate that these low specific activities were not constant values but could be slowly increased. The loss involved in the fractionation was so large as to render the process impractical as a method of preparation. It is evident, however, that occasionally beef pancreatic juice contains a protein which cannot be conveniently fractionated out by the procedure usually followed. There is an indication that the more completely the pancreas is extracted the more of this troublesome protein is contained in the extract. No active material could be isolated from *fresh* (inactive) beef pancreas, so that in the other preparations spontaneous activation had undoubtedly occurred.

### *Tests of Purity*

The fractionation experiment just described shows that the crystalline protein retains its constant chemical and physical properties, including its proteolytic activity, through a long series of successive fractionations and heatings. In these experiments, however, the principal fraction was analyzed. A much more sensitive test for the purity of a material consists in the comparison of the properties of the first small amount of precipitate obtained in a fractionation experiment with the properties of the final fraction left in the mother liquor after the bulk of the material has been precipitated. Theoretically, in either a mixture or a solid solution the maximum difference in composition would occur between these two fractions (21). A special series of experiments was therefore carried out in which a solution of the crystalline material was precipitated with ammonium sulfate and the specific proteolytic activity and optical activity of the first precipitate formed compared with that of the last small amount of material left in solution in the mother liquor. The results of this experiment are shown in Table IX.<sup>4</sup> The experiment shows that the

<sup>4</sup>The crystals as obtained directly always contain some nitrogen which is soluble in 2.5 per cent trichloroacetic acid. This may be removed by repeated

TABLE IX

*Fractional Precipitation with Ammonium Sulfate*

80 gm. crystalline trypsin filter cake dissolved in 500 ml.  $\frac{1}{4}$  saturated ammonium sulfate and precipitated by bringing ammonium sulfate concentration to 0.6 saturation. Repeated three times and precipitate washed with 0.7 saturated ammonium sulfate yield 30.0 gm. filter cake.

		Precipitate	[T. U.] <sup>4gV</sup> mg. P. N.	$[\alpha]_{25^\circ\text{C.}}^D$ mg. P. N.
		gm.		
7.5 gm. filter cake dissolved in 100 ml. $\frac{1}{4}$ saturated ammonium sulfate. Saturated ammonium sulfate added to slight precipitation. Filtered	Precipitate 1a	0.5	87	-0.235
Filtrate, saturated ammonium sulfate added until nearly all precipitated, filtered	Precipitate 1b	6.0	110	-0.258
	Mother Liquor 1c	1.0	119	-0.350
Precipitate 1b dissolved in 50 ml. $\frac{1}{4}$ saturated ammonium sulfate. Saturated ammonium sulfate added to slight precipitation, filtered	Precipitate 2a	0.1	103	-0.246
Filtrate, saturated ammonium sulfate added to heavy precipitate, filtered	Precipitate 2b	4.0	109	-0.257
	Mother Liquor 2c	1.5	124	-0.267
Precipitate 2b dissolved in 40 ml. $\frac{1}{4}$ saturated ammonium sulfate. Saturated ammonium sulfate added to slight precipitate, filtered	Precipitate 3a	0.3	95	-0.272
Filtrate, saturated ammonium sulfate added to heavy precipitate, filtered	Precipitate 3b	3.3	104	-0.267
	Mother Liquor 3c	0.3	106	-0.290

first small amount of precipitate obtained was slightly less active, but succeeding fractionations show no significant differences in either the

extraction in saturated magnesium sulfate or  $\frac{1}{4}$  saturated ammonium sulfate. On standing this soluble nitrogen again appears so that it is necessary to use freshly fractionated material for any experiment in which the purity is to be tested.

optical activity or proteolytic activity; *i.e.*, no evidence even under these extreme conditions was found to indicate that the material was either a mixture or a solid solution. They show quite conclusively that it is not a mixture or an adsorption complex of a protein with a non-protein molecule since in either case the composition of the first fraction would be quite different from that of the small amount of material remaining in the mother liquor. If the material were an adsorption complex the first small amount of protein precipitate would be expected to carry down relatively more of the adsorbed compound since this is a general property of adsorption systems. If it were a mixture the hypothetical active non-protein molecule would certainly have a different solubility in ammonium sulfate from that of the protein. In either case the composition of the extreme fractions obtained would be quite different even though the difference in solubility were slight. If the material, however, is a solid solution it is quite possible that no indication of this fact would be obtained in this type of experiment. However, since the bulk of the material is undoubtedly a protein it is unlikely that a solid solution would be formed except with another protein. The existence of a solid solution is rendered more unlikely by the fact that the optical activity as well as the proteolytic activity is constant since the optical activity is a very specific property even of similar proteins.

### *Solubility Experiments*

Another sensitive test for the purity of a protein consists in the determination of its solubility in the presence of varying amounts of solid phase. According to the phase rule, the solubility of a pure substance at constant temperature and pressure is constant and independent of the quantity of solid phase present, while the solubility of a mixture will increase at first with the quantity of solid present and will not be independent of this quantity until all the components present in the original solid are present in the solid phase. In the case of an ideal solid solution the solubility will increase gradually and indefinitely with increase in the quantity of solid present. The force of this method has already been illustrated in the case of the first crystalline protein obtained, since such experiments showed at once that the substance was not pure. A still more striking example is furnished

by Sørensen's experiments (22) with serum globulin and casein in which it was shown that these proteins although considered as chemical individuals for many years were not pure substances but were probably solid solutions of several similar proteins.

Unfortunately, owing to the unstable nature of the protein, it is impossible to carry out accurate solubility determinations with trypsin since even at 5°C. there is always some loss in activity during the course of the experiment. Another difficulty is that the solubility of the protein in ammonium sulfate is very sensitive to slight changes in the concentration of ammonium sulfate so that a difference of about 10 per cent in the ammonium sulfate concentration makes a difference of over 100 per cent in the solubility. This change in solubility with ammonium sulfate concentration is shown in Table X.

TABLE X

*Solubility Crystalline Trypsin in Different Concentrations Ammonium Sulfate in N/10 pH 4.0 Acetate Buffer at 5°C.*

m/10 pH 4.0 acetate buffer, ml.....	3	4	6
3.00 molar ammonium sulfate, ml.....	10	10	10
Ammonium sulfate concentration mols/liter.....	2.30	2.14	1.87
Protein nitrogen per ml., mg.....	0.30	0.74	>5.0

A good deal of time was spent in the attempt to obtain accurate solubility values but it was impossible to reach equilibrium conditions without some loss in activity. The experiments were made under the same general conditions as those already described for pepsin. The result of one such experiment is shown in Fig. 2 and represents the solubility of crystalline trypsin in a solvent prepared by mixing 4 ml. of N/10 pH 4.0 acetate buffer with 10 ml. 3 molar ammonium sulfate at 5°C. The points represented by open circles were obtained by dissolving the protein in the acetate buffer and precipitating the solution by adding ammonium sulfate; the points indicated by dots were obtained by stirring an excess of solid with the solvent. The fact that the values obtained in this way are about the same shows that the value is an equilibrium one. There is some indication that the solubility increases slightly with increasing quantity of precipitate but it is doubtful whether this increase is outside the experimental error.

The solid line in the figure represents the theoretical solubility curve for a substance having a solubility of 0.75 mg. protein nitrogen per ml., and a specific proteolytic activity, as measured by the change in viscosity of pH 4.0 gelatin of 100 T. U. per milligram protein nitrogen. The fact that the points indicating the activity per milliliter fall slightly below those indicating the milligrams protein nitrogen per milliliter shows that the specific activity, as determined in this particular experiment, was slightly less than 100. Determinations made with the same material somewhat later and with a new gelatin solution gave a value for the specific activity of 100 so that this low figure was probably due to a slight difference in the gelatin preparation.

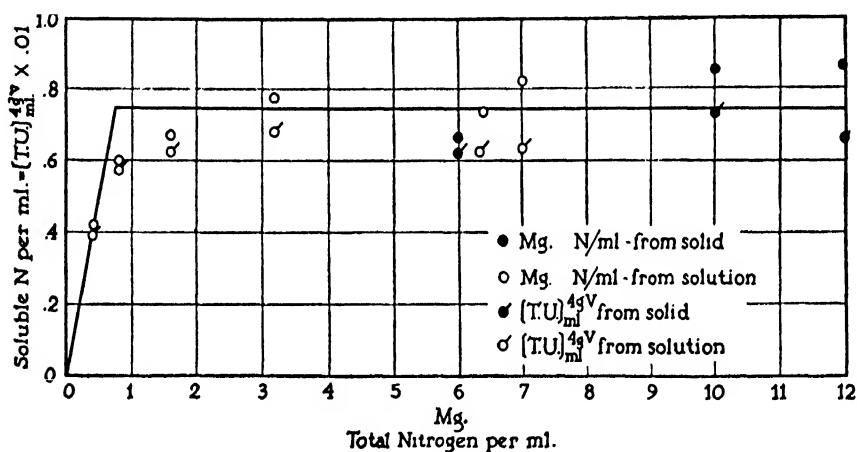


FIG. 2. Solubility of crystalline trypsin in  $\left\{ \begin{array}{l} 4 \text{ ml. N/10 pH 4.0 acetate buffer} \\ 10 \text{ ml. 3 m. ammonium sulfate} \end{array} \right.$  at 5°C. with increasing quantities of solid trypsin.

A probable cause of diverging results in these experiments is the difference in solubility between the crystalline and amorphous forms of the precipitate. In the case of pepsin (9) this complicating effect could be clearly detected and experimentally verified by microscopic examination of the precipitate. In the case of trypsin, however, the crystals are so small as to render it difficult under the conditions of a solubility experiment to determine microscopically whether the precipitate is crystalline or amorphous. The determinations made with a total of 6 mg. total nitrogen per ml. were obtained with a precipitate which was undoubtedly crystalline and since they fall in

the same concentration range as the others, it is probable that the precipitate in all these experiments was crystalline since a marked difference in solubility between the crystalline and amorphous forms is generally found with proteins. As a whole the fractionation experiments show quite conclusively that the crystalline material is not a mixture nor an adsorption complex but they do not rule out the possibility that the material is a solid solution.

*Relation of the Proteolytic Activity to the Protein*

*Heat Inactivation.*—The experiments described show that under the conditions studied so far the material behaves like a pure substance, or

TABLE XI

*Decrease in Activity and Protein Concentration on Heating Trypsin Solutions in N/10 Hydrochloric Acid*

2 gm. crystalline filter cake dissolved in 40 ml. N/10 hydrochloric acid and solution heated to 95°C. by immersion in boiling water. 1 ml. samples taken, cooled to 0°C. for 2 minutes, and added to 4 ml. 0.7 saturated magnesium sulfate in N/1 sulfuric acid. Precipitate formed. Centrifuged and protein nitrogen and activity determined on supernatant liquid.

Time at 95°C., hrs.....	0	0.10	0.20	0.30	0.40
[T. U.] $\frac{4gV}{ml.}$ .....	118.0	32.8	22.4	7.0	0.94
Protein nitrogen per ml., mg.....	1.50	0.315	0.245	0.245	0.105
[T. U.] $\frac{4gV}{mg. P. N.}$ .....	80	104	92	29	9.0

possibly a solid solution. They indicate, therefore, that the proteolytic power of the preparation is a property of the protein molecule. This conclusion may be tested in a number of other ways. If the proteolytic activity is a property of the protein molecule then any chemical change in the protein should be reflected by a change in activity. When a solution of the protein is heated for a short time, the protein is denatured; *i.e.*, it is changed to a form which is precipitated by low concentrations of salt. At the same time the activity is lost. When the solution is allowed to cool, however, the protein is no longer precipitated by salt but reverts to its original native condition; at the same time its original activity is regained. This reaction has been studied in detail and



reported in another paper (16). If a solution of the protein is heated for a longer time, however, it does not return to its original soluble condition on cooling nor does the activity return. There is evidently a second non-reversible reaction which changes the denatured protein into another form. If the rate of formation of this permanently denatured protein be compared with the loss in activity of the solution, it is found that the decrease in activity is proportional to the decrease in native protein. The results of such an experiment are shown in Table XI. This experiment is carried out in the same way as that already reported with the first crystalline material obtained (Table III). In that case the specific activity increased rapidly at first, showing that the original material undoubtedly contained an inactive protein. In the present experiment there is also a slight increase in the specific activity at first. The specific activity then remains practically constant until only 20 per cent of the original total activity is left; it then decreases rapidly. Evidently on long heating there is formed in the solution a compound which does not precipitate with salt solution but does precipitate with trichloroacetic acid. Such compounds are always found in the acid hydrolysis of proteins and it is not surprising that they are found under these conditions. The material used in this experiment originally had a specific activity of slightly more than 100. On standing the activity decreased somewhat, evidently with formation of a protein which is rapidly and permanently denatured by heat, so that the first few minutes heating results in a slight increase in the specific activity to its original value. This behavior has been noted consistently and indicates that on standing at room temperature the active protein becomes transformed into an inactive one which has lost the remarkable property of reverting to the native condition immediately on cooling after being heated. This experiment shows that when the protein is denatured by prolonged heating there is a corresponding loss in activity and therefore agrees with the result expected if the activity were a property of the protein molecule.

*Pepsin Digestion.*—It is known that pepsin digestion destroys the tryptic activity of a solution (Long and Johnson; Long and Hull (23)). If the proteolytic activity were a property of the native protein molecule it would be expected that the loss in activity during pepsin digestion would be proportional to the loss of native protein. If, on the

other hand, the proteolytic activity were due to some molecular species accompanying the protein, it would be expected that the protein concentration would decrease more rapidly than the activity since, so far as is known, pepsin acts only on proteins. This result would also be expected if the material were a mixture of an inactive with an active protein, since the rate of hydrolysis of different proteins with pepsin is highly specific. This would result in a change in the specific activity when calculated on the basis of total protein present. The same result would be obtained if any portion of the protein molecule

TABLE XII

*Decrease in Activity and Protein Concentration in Trypsin Solutions Digested by Pepsin*

1 gm. crystalline trypsin cake dissolved in 30 ml.  $M/100$  hydrochloric acid at  $35.5^{\circ}C$ . 0.5 ml. crystalline pepsin solution containing 0.28 mg. protein nitrogen added. Protein nitrogen determined with 2.5 and 18 per cent trichloroacetic acid. Activity determined by gelatin viscosity method. The activity due to pepsin was negligible.

Time at $35^{\circ}C$ ., hrs.....	0	1	2	4	8	24	48
[T. U.] <sup>4gV</sup> ml. ....	125	99	74	56	40	20	10.3
Protein nitrogen per ml., mg.							
18 per cent $CCl_3COOH$ .....	1.49	1.24	1.1	0.96	0.79	0.51	0.40
2.5 " " $CCl_3COOH$ .....	1.21	0.93	0.62	0.51	0.40	0.23	0.20
[T. U.] <sup>4gV</sup> mg. P. N.							
18 per cent $CCl_3COOH$ .....	84	80	67	58	51	39	26
2.5 " " $CCl_3COOH$ .....	103	107	119	110	100	87	52

retained any appreciable activity. In this case the specific activity of the total protein left in solution would increase since the activity due to the fragment of the protein molecule would be added to that due to the unchanged protein itself. The result of an experiment in which crystalline trypsin was digested with a very small quantity of crystalline pepsin is shown in Table XII. The total protein present in solution was determined by precipitation with 2.5 per cent and also with 18 per cent trichloroacetic acid. No quantitative method exists by which the native protein alone can be determined but the nitrogen precipitated by 2.5 per cent trichloroacetic acid is probably not far

from the correct amount. 18 per cent trichloroacetic acid precipitates considerably more nitrogen than 2.5 per cent and if any of the higher split products of the protein retained any activity, it might be expected that the specific activity calculated from nitrogen precipitated with 18 per cent trichloroacetic acid would be more constant than that obtained when 2.5 per cent trichloroacetic acid is used. The results of the experiment, however, show that the specific activity calculated on the basis of the nitrogen precipitated with 2.5 per cent trichloroacetic acid remains constant until more than 70 per cent of the total activity is lost, while the specific activity calculated on the basis of 18 per cent trichloroacetic acid decreases rapidly. Apparently, therefore, as soon as the protein is split so that it no longer precipitates with 2.5 per cent trichloroacetic acid all its activity is lost and even the fragments still large enough to precipitate with 18 per cent trichloroacetic acid possess no activity. The results indicate that the proteolytic activity is a property of the entire molecule as is the case with the (reversible) oxygen combining power of hemoglobin. It was found that this experiment was a very sensitive test for the purity of the preparation used, as is the effect of heating in acid. Preparations which have become partially inactivated show at first a rapid increase in the specific activity under these conditions due to the fact that the inactivated, denatured protein is more rapidly hydrolyzed than the active, native protein. With such preparations, therefore the initial specific activity is low but increases rapidly until it reaches a figure of about 100 and then remains constant.

#### *Alkali Inactivation*

When a solution of trypsin is allowed to stand in slightly alkaline solution the protein decomposes rapidly (16) so that these conditions furnish another method of comparing changes in the protein and changes in the activity. The results of an experiment under these conditions are shown in Table XIII. They are similar to those obtained with pepsin digestion in that the decrease in activity is very nearly proportional to the decrease in protein nitrogen as determined by 2.5 per cent trichloroacetic acid, and is greater than the loss in protein nitrogen as determined by 18 per cent trichloroacetic acid. They indicate again that the proteolytic property is lost so soon as any change occurs in the original native protein molecule.

The hydrolysis of the protein under these conditions may be ascribed to several possible mechanisms. It may be simply a case of the usual alkali hydrolysis of proteins, or it may be considered that the protein digests itself, and in either case it might be supposed that the formation of denatured protein was an intermediate step in the reaction so that the experiment as a whole might be considered simply as a variation of the result already obtained (16) to the effect that the activity is lost when the protein is denatured.

TABLE XIII

*Decrease in Activity and Protein Nitrogen Concentration of Trypsin Solutions in Sodium Bicarbonate at 35.5°C.*

0.50 gm. crystalline trypsin filter cake dissolved in 15 ml. N/100 sodium bicarbonate at 35.5°C. Protein nitrogen determined by precipitation with 2.5 or 18 per cent trichloroacetic acid. Activity determined by viscosity pH 4.0 gelatin.

Time at 35.5°C., hrs.....	0	0.5	1.0
Protein nitrogen per { 18 per cent CCl <sub>3</sub> COOH), mg.....	1.0	0.66	0.54
ml. solution { 2.5 " " CCl <sub>3</sub> COOH), mg.....	0.85	0.46	0.40
[T. U.] <sup>4gV</sup> / <sub>ml.</sub> .....	116	52	34
[T. U.] <sup>4gV</sup> / <sub>mg. P. N.</sub> { 18 per cent CCl <sub>3</sub> COOH.....	116	79	62
{ 2.5 " " CCl <sub>3</sub> COOH.....	(136) <sup>1</sup>	113	84

<sup>1</sup> This figure is based on one experimental value and is probably an experimental error.

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# CRYSTALLINE TRYPSIN

## II. GENERAL PROPERTIES

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The isolation of a crystalline protein having constant properties, including powerful proteolytic activity, was described in the preceding paper (1). Experiments were also described which showed that destruction of the protein by any method tried resulted in a corresponding loss in proteolytic activity. The present paper describes some of the general properties of the preparation.

### *Extent of Hydrolysis*

It was pointed out in discussing the method of preparation, that, as purification is continued, the extent to which the hydrolysis is carried decreases rapidly and in fractions after No. 5 the hydrolysis stops at an increase in formol titration of about 9 ml. N/50 per 5 ml. 5 per cent protein solution for casein and 7 ml. for gelatin. This figure is independent of the quantity of the enzyme used and also of the time over which it acts. In other words, it is a final equilibrium value. This figure represents very nearly an increase of 100 per cent in the number of free carboxyl (or amino) groups of casein and of 200 per cent for gelatin and shows that the action of this purified enzyme consists in splitting the molecule once for each free amino (or carboxyl) group already present in casein, and twice in gelatin. The data for an experiment in which this maximum hydrolysis was determined are shown in Fig. 1. It will be noted that this total increase corresponds approximately to that defined by Willstätter (2) as one tryptic unit. It is evident, therefore, that the method of determining tryptic activity used by Willstätter and his collaborators cannot be used for the proteolytic enzyme obtained in the present experiment since the digestion

follows an entirely different course with the purified enzyme from that of the original crude material. This applies to the form of the curve obtained as well as to the final value. This result has previously been reported by Schönfeld-Reiner (3).

Fig. 1 is plotted on a small scale so that the initial stage of the reaction does not appear. In Fig. 2 the first part of the reaction is plotted

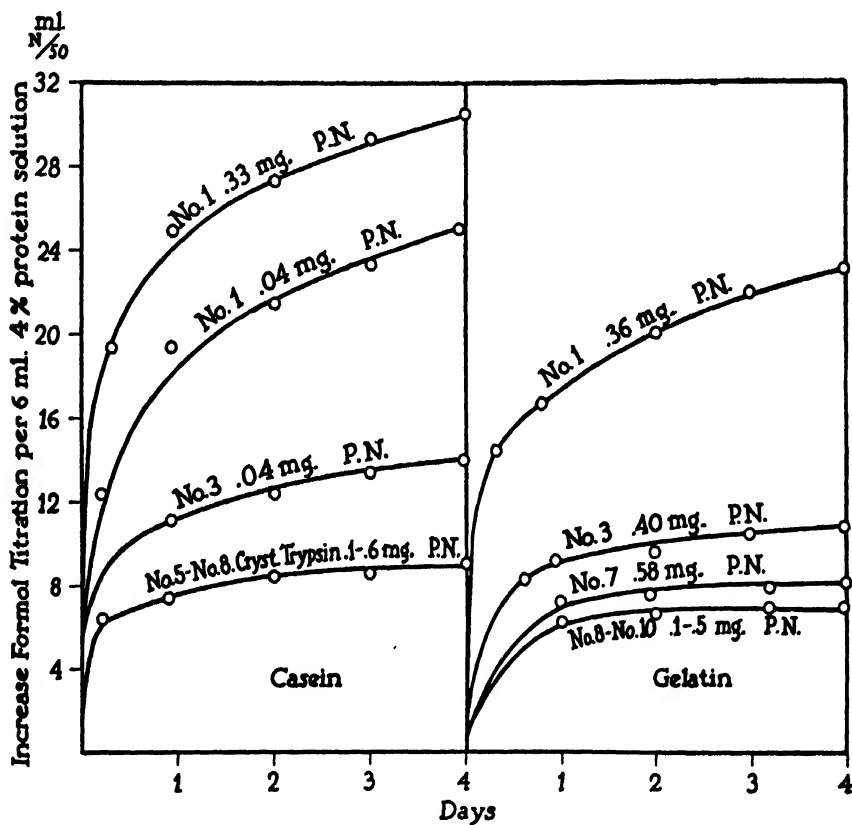


FIG. 1

on a large scale. The figure shows the striking difference in the form of the reaction curve as determined with crystalline or crude trypsin, either by formol titration or the production of non-protein nitrogen. Since the curves obtained with the crude and crystalline fraction cross in two points, it is evident that they cannot be made to coincide throughout. All the curves, however, are straight lines, within the experimental error, up to an increase in formal titration of about 1 ml.

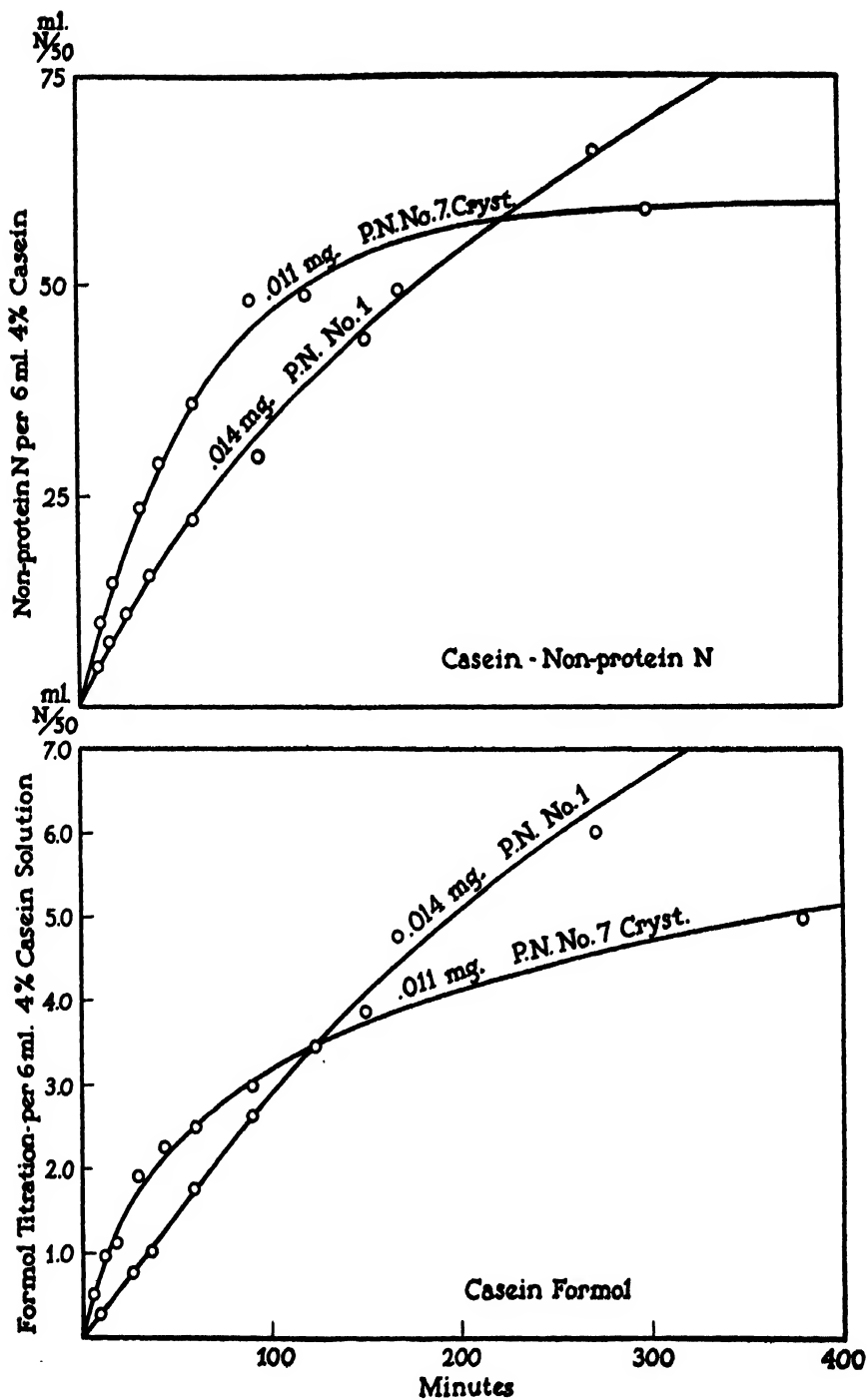


FIG. 2



or an increase in soluble nitrogen corresponding to an increase of about 5 ml.  $N/50$  alkali, and in this part of the reaction therefore the curves may be superimposed. It follows from this that the curve obtained with the crude material cannot be used to measure the activity of the purified fraction except over this very small initial part of the curve. If the part of the curve beyond this point is used, it will be found that a different figure for the activity of the preparation will be obtained with each different concentration so that the figure is meaningless.

The effect of the protein concentration on the rate of reaction also varies with the purity of the enzyme (Northrop (4)).

The extent of hydrolysis of casein with trypsin-kinase, as reported by Abderhalden and Schwab (5), Waldschmidt-Leitz and Simons (6) is much greater than that caused by the crystalline trypsin and agrees approximately with that found with the crude fractions. This result shows that the enzyme isolated in the course of this work is different from that described by Waldschmidt-Leitz. It is possible that the two enzymes are entirely distinct but it seems more probably that the trypsin-kinase still contains some of the peptonases. The fact that more than half of the original protease activity is contained in the final crystalline fraction shows that the crystalline material represents the principal protease present in the crude material.

The fact that the crude preparation carries the hydrolysis much further than does the purified enzyme might be due to the fact that there is present in the crude material some substance which acts as a coenzyme and which enables the trypsin to attack the primary product resulting from the hydrolysis of the protein. An effect similar to this has been described by Abderhalden (7) who found that enzyme fractions which could not hydrolyze some polypeptides acquired the ability to hydrolyze these substances when some of the decomposition products were added to the digestion mixture. If this mechanism were correct it would be expected that the action of the purified and crude material mixed together would be greater than the sum of the two actions separately and also that the addition of heated crude material to the pure enzyme would allow digestion to proceed further than with the pure enzyme alone, since such coenzymes are usually heat stable. If, on the other hand, the extent of hydrolysis were due to the fact that the crude material contains one or more enzymes

capable of attacking the products formed by the trypsin itself, it would be expected that the action of the purified enzyme should be independent of the presence of the crude fraction. These possibilities were tested by adding crude and crystalline trypsin to a solution of gelatin which had already been digested with the crystalline enzyme, and which had then been boiled to inactivate any enzyme remaining. The results of this experiment are shown in Table I. The experiment shows that the crystalline trypsin is unable to carry digestion any further, even when the inactive, crude preparation is added, and

TABLE I

*Extent of Hydrolysis of Gelatin with Mixtures of Crude and Crystalline Trypsin*

Crystalline trypsin solution 0.005 mg. protein nitrogen per ml.	0.5 [T. U.]	$\frac{4gV}{ml.}$				
Crude trypsin solution 0.025 mg. protein nitrogen per ml.	0.5 [T. U.]	$\frac{4gV}{ml.}$				
Gelatin—5 per cent pH 7.6 in M/10 7.6 phosphate buffer						
150 ml. gelatin solution + 5 ml. crystalline trypsin solution; 35.5°C. for 48 hrs.; boiled $\frac{1}{4}$ hr.						
Digestion mixture made up as follows:						
Gelatin solution (boiled), ml. ....	10	10	10	10	10	10
Crystalline trypsin solution, ml. ....	0	0.1	0	0	0.1	0.1
Active crude trypsin, ml. ....	0	0	0	0.1	0.1	0
Boiled crude trypsin, ml. ....	0	0	0.1		0	0.1
Formol titration (ml. N/50 NaOH) per 5						
ml. at 35°C. after 20 hrs., ml. ....	0	0	0	3.0	3.05	0.05
After 48 hrs. ....	0.10	0.15	0.05	4.8	4.70	0.10

also that the rate at which the digestion proceeds with the crude preparation is not increased by the addition of the crystalline trypsin. The results as a whole, therefore, indicate that the difference in the extent of digestion is due to the presence of several enzymes in the crude material rather than to any activation effect of the crude material on the trypsin itself.

#### *Hydrolysis by Trypsin Following Pepsin*

The crystalline trypsin hydrolyzes casein less than does pepsin, and gelatin to about the same extent but the hydrolysis occurs at different linkages with the two enzymes. This is shown by the fact

that gelatin or casein solutions, previously digested by pepsin, are hydrolyzed to the same extent, with crystalline trypsin, as are the original protein solutions.

A summary of an experiment in which casein and gelatin were first digested in acid solution with an excess of crystalline pepsin and then digested at pH 7.6 with crystalline trypsin is shown in Table II. The solutions in every case were titrated first to about pH 7.4 and the formol titration determined from this point so that the figures are comparable. The table shows that pepsin or trypsin alone very nearly triples the formol titration of gelatin and also that trypsin digestion following pepsin causes nearly the same increase as when trypsin acts on the original protein solution. Either enzyme, therefore, triples

TABLE II

*Hydrolysis of Gelatin and Casein with Crystalline Pepsin and Trypsin*

Formol titration per 5 ml. 5 per cent protein solution, pH 7.0; ml. N/50 sodium hydroxide.

Protein	Casein total	Increase	Gelatin total	Increase
Original protein solution.....	9.0		4.0	
After pepsin digestion alone.....	27.0	18.0	11.5	7.5
After trypsin digestion alone.....	18.0	9.0	11.0	7.0
After pepsin digestion followed by trypsin....	36.0	27.0	19.0	15.0

the number of titrable groups present so that there must be two hydrolyses for each original carboxyl group. Since gelatin originally contains about 30 acid groups per molecule, as determined by this method of titration, hydrolysis by either pepsin or trypsin alone must take place in about 60 places in the gelatin molecule. The two enzymes together, therefore, cause hydrolysis at about 120 groups. The formol titration of casein solutions is only doubled by trypsin digestion so that with this protein hydrolysis occurs at about 100 places in the molecule since there are about 100 titrable groups per mole of casein. This is less than the increase caused by pepsin alone, which corresponds to an increase of 200, or 200 per cent in the number of titrable groups. The action of both enzymes on casein liberates about 300 new groups from the casein molecule.

Crude trypsin preparations carry the digestion of such peptone solutions at least three times as far as does the crystalline trypsin.

*Effect of Enterokinase on the Activity of the Crystalline Trypsin*

Trypsin is known to exist in the pancreas in an inactive form, named trypsinogen. The active enzyme appears when the trypsinogen is mixed with intestinal juice or tissue extract. This activating effect of intestinal juice is assumed to be due to the presence of a substance called enterokinase. It is also known that trypsin becomes active if

TABLE III

*Effect of Enterokinase on Glycerine Extract of Dried Pancreas and on a Solution of Crystalline Trypsin*

Glycerin extract prepared according to Willstätter, diluted 1/10. Kinase solution after Waldschmidt-Leitz. 1 ml. enzyme solution + 4 ml. kinase, 30 minutes at 30°C. Activity determined.

	Kinase solution		Original enzyme solution						
			N/ml.	[T. U.] <sub>ml.</sub>			[T. U.] <sub>mg. P. N.</sub>		
	Active	Inactive			4.7 gV	Cas. F.	Cas. S	4.7 gV	Cas. F
		ml.	ml.	mg.					
Glycerin extract	0	4	0.21						
	2	2	0.21	23.0	0.008	0.09	119	0.042	0.45
Crystalline trypsin	0	4	0.0084	7.5	0.0014	0.017	900	0.17	2.05
	2	2		7.7	0.00135	0.015	920	0.16	1.8
	4	0		6.3	0.00059	0.015	750	0.07	1.8
	0	0		7.7	0.0014	0.017	900	0.17	2.05

chopped pancreas or pancreatic extract is allowed to stand, especially in slightly acid solution.

The raw material used in the present work was obtained from pancreas which had stood for some time and it is probable that activation had already occurred. No attempts to activate it were made during the activity determinations since this procedure would undoubtedly lead to the isolation of an unknown mixture of active plus inactive enzyme. The activity of the crystalline enzyme is not increased by the addition of enterokinase as is shown by the experiment reported in Table III.

The kinase and the glycerin pancreatic extract were prepared according to Willstätter and Waldschmidt-Leitz (9) and their conditions for activation were used (2). The composition of the solutions for activation is given in the left side of the table. The volume was made up to 5 ml. in each case with kinase solution which had been boiled in order to keep conditions as nearly constant as possible. The experiments with glycerin extract show that the kinase was active. Control experiments showed that the boiled kinase did not interfere with the reaction, and also that neither active kinase nor boiled kinase alone has any effect on the methods of determining activity. The activation mixture was kept for  $\frac{1}{2}$  hour at 30°C. and then analyzed for activity by the usual methods. The resulting specific activity has been expressed as the number of activity units (8) per milligram protein nitrogen of the original enzyme solution. The results show that the kinase brought the specific activity of the glycerin extract up to about that of the pancreatic extract used as a starting point in this work. The specific activity of the crystalline trypsin is not affected by the addition of the kinase.

Since, according to Waldschmidt-Leitz (10) the reaction between kinase and trypsin is stoichiometric, it is necessary to be sure that there is an excess of kinase present since the addition of a small amount of kinase to a mixture already containing a large amount of active enzyme would show no effect. The quantity of active enzyme used in this experiment, therefore, is the minimum quantity which could be detected by the methods used and represents about the same order of activity as that of the activated glycerin extract.

#### *Effect of pH on the Rate of Digestion of Casein by Trypsin*

The rate of digestion of a 5 per cent casein solution titrated to various pH and at 35.5°C. was determined by following the formation of nitrogen not precipitated by 5 per cent trichloroacetic acid at different time intervals. The initial rate of the reaction was determined from the slope of these curves. The result of the experiment is shown in Fig. 3 in which the maximum rate observed is taken as 100 per cent. For comparison determinations made previously with crude trypsin preparations are included (11). There is no marked difference between the two curves and both show a rather flat maximum extending from about pH 7.5 to 9.5.

### *General Protein Tests*

A 0.5 per cent solution of the material gave positive tests with Biuret, Millon, xanthoproteic, and Folin's tyrosine reagent (12).

In 0.0005 per cent solution, which is approximately the concentration used for activity determinations, all the protein tests were negative. It is therefore perfectly possible to have active solutions or preparations of the enzyme which give no protein reaction, simply because they are too dilute.

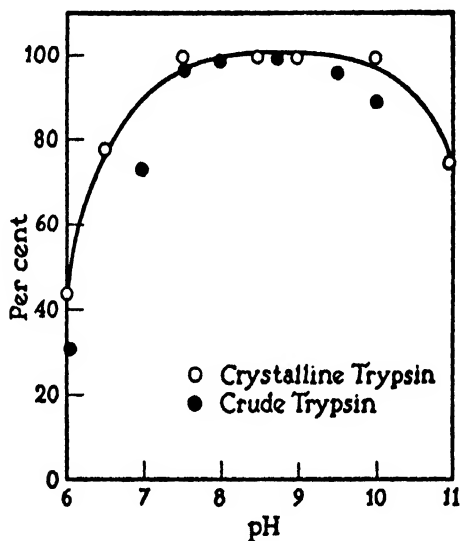


FIG. 3

### *Number of Carboxyl Groups*

0.137 gm. of the protein dissolved in 5 ml. water (pH 8.0) required 7.0 ml.  $N/50$  alkali for formol titration. This corresponds to 0.001 equivalents of amino (or carboxyl) groups per gm., or 34 per mole.

### *Molecular Weight*

The molecular weight of the protein was determined by osmotic pressure measurements in  $\frac{1}{4}$  saturated ammonium sulfate and pH 4.0 acetate buffer. The experiments were done with different concentrations of the protein and the pressure was read some time after it remained constant. The results of the experiment are shown in Table IV. The molecular weight is about 34,000.

*Determination of the Diffusion Coefficient<sup>1</sup>*

The determination was carried out as described by Northrop and Anson (13) and the quantity of material diffusing determined both by total nitrogen and by proteolytic activity. In the case of Cell 2 less

TABLE IV  
*Osmotic Pressure*

20 gm. crystalline trypsin filter cake dissolved in  $\frac{1}{2}$  saturated ammonium sulfate pH 4.0 acetate buffer. Solution put in rocking osmometer in same solvent at 5°C. Osmotic pressure determined after 24 hours. Solution analyzed for protein nitrogen.

Mg. protein per ml. = $\frac{\text{mg. N}}{\text{ml.}} \times 6.5 \dots\dots$	72	71	50	49	22.5	18
Pressure, mm. Hg = $P \dots\dots\dots$	39	38	22.0	21.5	11	9
Molecular weight $\frac{278 \times 760 \times 22.4}{273 P} \times \text{gm.}$						
protein/l. $\dots\dots\dots$	32,000	32,300	39,300	39,500	34,600	34,600

TABLE V  
*Diffusion Constant of Crystalline Trypsin*

Cell No. $\dots\dots\dots$	2	4	2	4
Cell constant (hemoglobin standard) $\dots\dots\dots$	0.054	0.0315	0.054	0.0315
Solvent $\dots\dots\dots$	0.5 saturated magnesium sulfate N/10 acetate buffer pH 4.0		0.5 saturated magnesium sulfate sulfuric acid pH 3.0	
$\eta$ solvent 5°C. erg sec. cm. <sup>-2</sup> $\dots\dots\dots$	0.0303	0.0303	0.0362	0.0362
Sp.g. solvent 5°C. gm. cm. <sup>-3</sup> $\dots\dots\dots$	1.115	1.115	1.145	1.145
Average $D$ cm. <sup>2</sup> day <sup>-1</sup> $\dots\dots\dots$				
nitrogen $\dots\dots\dots$	0.0207 $\pm$ 0.001		0.0187 $\pm$ 0.001	
activity $\dots\dots\dots$	0.0218 $\pm$ 0.001		0.020 $\pm$ 0.002	
Radius trypsin molecules. $\dots\dots\dots$	$2.72 \times 10^{-7}$ cm.		$2.5 \times 10^{-7}$ cm.	

than 10 per cent of the dissolved protein diffused out during the course of the experiment, while with Cell 4 about 25 per cent diffused out. The results of the experiment are shown in Table V. The value for

<sup>1</sup> These experiments were carried out by Dr. Henry W. Scherp.

the diffusion coefficient is the same within the experimental error for both cells and for both methods of determination.

This is direct experimental evidence that the molecules responsible for the proteolytic activity are the same size as those which contain the protein nitrogen and that the enzyme molecule cannot be separated from the protein molecule by fractional diffusion. The average value of the diffusion coefficient is  $0.020 \pm 0.001$  cm.<sup>2</sup> per day, corresponding to a molecular radius of about  $2.6 \times 10^{-7}$  cm.

The volume of 1 mole of (hydrated) protein is therefore  $(2.6 \times 10^{-7})^3 \times 4/3\pi \times 6.06 \times 10^{23} = 44,700$  cm.<sup>3</sup> From the osmotic pressure measurements the molecular weight of the anhydrous protein is 34,000. If the specific volume of anhydrous protein is assumed to be 0.75 (the value generally found for protein), this corresponds to a molecular volume of (anhydrous) protein of 26,200 ml. Therefore, each mole of protein carried with it about 18,500 gm. of water, which corresponds to about 0.53 gm. of water per gm. of anhydrous protein, and a molecular weight for the hydrated protein of 53,500.

#### *Hydration from Viscosity Measurements*

Hydration of trypsin was determined by measuring the viscosity of various concentrations of crystalline trypsin in 0.5 saturated magnesium sulfate pH 4.0 at 5°C. The results showed that the trypsin is hydrated to the extent of 0.5 gm. of water per gm. of dry trypsin and is independent of the concentration of trypsin in the range of 4.0 to 0.8 gm. of protein per 100 ml. of solution. The hydration value was calculated from the specific viscosity by means of Kunitz's formula (14). The specific gravity of the anhydrous protein was assumed to be 1.33. The value obtained in this way agrees very closely with that calculated above from the diffusion and osmotic pressure measurement.

#### *Isoelectric Point*

If a solution of the crystalline trypsin is added to boiling M/10 phosphate buffer of various pH, a precipitate is formed between pH 7.0 and 8.0. This precipitate undoubtedly consists of denatured protein and the relation of this zone of precipitation of the denatured protein to the isoelectric point of the native protein is therefore somewhat doubtful. Cataphoresis measurement showed that the isoelectric point of col-



lodon particles suspended in dilute solutions of trypsin in different buffers is about pH 6.0 in M/50 acetate, about pH 7.0 in M/50 phosphate, and between pH 5.0 and 6.0 in the presence of M/50 ammonium sulfate. These results indicate that the isoelectric point is in the neighborhood of pH 7.0 but its exact position can only be determined by transport measurements. Apparently, however, it is more towards the acid side than that found for crude trypsin preparations by means of the distribution of the enzyme in gelatin particles (Northrop (16)) which indicated an isoelectric point at about pH 10.0. Willstätter (17) also concluded from the behavior with various adsorbents that the enzyme with which he was working had an isoelectric point quite

TABLE VI

*Inactivation of Trypsin Solutions at 30°C. and Various pH*

About 5 per cent solution of crystalline trypsin made up in  $\frac{1}{4}$  saturated ammonium sulfate and increasing amounts of sulfuric acid. pH measured and activity determined after various time intervals at 35°C.

Final concentration H <sub>2</sub> SO <sub>4</sub> .....	N/4	N/8	N/16	N/32	N/64	N/128	N/256	N/512	N/1024
pH (electrometric)...	1.5	1.8	2.16	2.42	2.64	2.81	2.95	3.0	3.12
Time at 30°C.	Per cent of original activity after increasing time at 30°C.								
hrs.									
46	81	100	95	80	60	50	48	44	31
70	67	76	74	62	39	35	30	26	26

far on the alkaline side. The enzyme described in this paper, however, is undoubtedly different from that with which Willstätter's experiments were done.

*The Effect of pH on the Stability of Trypsin*

The rate of inactivation of a solution of crystalline enzyme in  $\frac{1}{4}$  saturated ammonium sulfate at 30°C. was determined. The activity determinations were made by the gelatin viscosity method. The results of the experiment are shown in Table VI. There is a rather sharp maximum for stability at about pH 2 under these conditions.

The results in general are quite different from those obtained by the writer (26) and by Pace (27) with crude trypsin preparations since the crude preparations were found to be most stable at about pH 6.0.

## DISCUSSION

The experiments described in these papers show that a crystalline protein may be isolated from pancreas which has constant physical and chemical properties including intense proteolytic activity. The protein has been studied under a variety of conditions which would be expected to show evidence of mixtures without causing any demonstrable change in its characteristic properties. If the material were other than a protein these experiments would justify the statement that it was a pure substance. Since it is a protein, however, it is quite possible that the material may be a solid solution, as in the case of proteins such solid solutions frequently exist and are extremely difficult to fractionate into their components. The problem is rendered unusually difficult in this case by the extremely unstable nature of the protein. It seems unlikely, however, that the material contains any non-protein molecular species. The constant composition under various conditions of fractionation precludes the possibility of an adsorption compound since it is characteristic of these compounds that their composition varies with the conditions of precipitation.

Even though the crystalline material is a mixture or solid solution and not a pure substance, there seems good reason to believe that the proteolytic activity and the protein properties are attributes of the same molecule. This conclusion is confirmed by a number of experiments in which it was found that any change in the protein properties caused a corresponding decrease in the activity of the solution. Denaturation of the protein by heat, hydrolysis by acid or pepsin or alkali all cause the concentration of native protein in the solution to decrease and this decrease is accompanied by a corresponding decrease in activity. In addition, the denatured, inactive, protein formed by heating the solution reverts to the native condition when the solution is cooled and at the same time the normal specific activity returns (18). In order to account for these results on the assumption that the activity is due to the presence of some non-protein molecule, it is necessary to assume that this hypothetical molecule cannot exist in the absence of the protein and also that it regains its activity under the same conditions as cause the denatured protein to return to the native form. In the absence of positive proof for the existence of such a hypothetical molecule these assumptions seem unlikely. So far as

the writer is aware, there is no positive proof of the existence of such molecules and the assumption that they exist rests merely on the negative fact that most of the attempts to prepare pure substances, *i.e.* those with constant properties including enzymatic activity, have been unsuccessful. On the other hand it is, of course, impossible to disprove the existence of such molecules. Since nothing is known of the properties of these hypothetical active molecules it would be perfectly logical to assume that they are proteins themselves, especially since the general properties of enzymes such as inactivation by heat, adsorption on surfaces, and destruction by strong acid or alkali are in general those of proteins.

Active enzyme preparations have been obtained which contain very small amounts of protein; on the other hand extremely active preparations of urease, pepsin, and trypsin, and amylase have been obtained which are pure, or nearly pure proteins. If it be assumed that the activity of these protein preparations is due to the presence of some minute amount of a non-protein molecule, it is equally reasonable to assume that the activity of the non-protein preparation is due to the presence of a minute amount of protein.

The fact that in other cases the enzymatic activity may vary independently of the total protein content of the preparation proves only that some of the protein present is inactive but not that all of it is inactive.

Numerous experiments have been reported in the literature in which solutions of pepsin and other enzymes have been found to give negative protein tests although they are active. These experiments are also inconclusive since the activity test is far more delicate than the chemical test for proteins. For instance, a solution of crystalline trypsin or pepsin containing less than 1/1,000,000 of a gram of protein nitrogen per milliliter has an accurately measurable effect on the digestion of casein, while solutions of pepsin containing less than 1/10,000,000 of a gram of nitrogen have a very powerful effect on the coagulation of milk. Such solutions give negative results with protein tests but the dry material from which the solutions are made is practically pure protein. The minimum concentrations of these enzymes which can be detected are at least ten times less than the concentrations mentioned above and are of the same order of magnitude as the concentration of

respiratory ferment in yeast as calculated by Warburg and Kubowitz (19).

It appears to the writers, therefore, that the assumption that enzymes are proteins is in the best accord with the facts up to the present time. Since these proteins possess characteristic enzymatic activity, in addition to the usual properties of proteins, they must possess some characteristic chemical structure which may or may not be an amino acid complex. The problem is the same as in the case of insulin (20). In general, most properties of molecules cannot be considered quantitatively as the sum of the properties of the various groups or atoms of which they are made, but must be considered as properties of the whole molecule. Thus, the optical activity, color, strength of acid groups, etc., of any one molecule depends qualitatively on the presence of a certain group or groups, but quantitatively, the property is affected by any change in the molecule. For instance, to possess optical activity a molecule must contain an asymmetric atom but its specific optical activity will change with any change in the molecule and it is impossible to isolate a group from the compound possessing the optical activity of the whole molecule. The same is true of the color of dyes to a more marked degree.

Hemoglobin presents perhaps the best example. This substance has the general properties of a protein but in addition possesses the remarkable property of combining reversibly with oxygen. It acts as a catalyst in certain oxidation reactions and might, therefore, be considered an enzyme. The property of combining reversibly with oxygen is assumed to be due to the presence of the iron-pyrrol group but denaturation of the protein, a reaction common to all proteins, destroys its power of combining reversibly with oxygen although the denatured protein still contains the prosthetic group.

Krebs (21) has shown that heme itself is a very poor catalyst but when combined with certain nitrogenous groups it forms hemochromogens, some of which are very effective catalysts. Thus the catalytic properties of hemoglobin and these related compounds are all due to the presence of the heme group, but this group when isolated has little or no catalytic activity and the catalytic power of the various heme compounds depends upon the substance with which the heme is combined. It is quite possible that the same general condition

applies to other enzymes and that there are an indefinite number of closely related enzymes depending upon the compound with which the characteristic group is combined. This point of view does not differ very much from that developed by Willstätter (22) and his collaborators except that it regards the various active compounds as definite chemical individuals rather than as adsorption complexes of varying composition.

At the present time, however, there is no direct evidence of the existence of any peculiar prosthetic group not found in other proteins and it is quite possible that their activity depends on some peculiar arrangement of the amino acids, as Jensen (20) has suggested in the case of insulin.

### *Mechanism of the Catalytic Effect*

Sörensen (23) has shown that protein solutions in the presence of the solid phase are in true equilibrium and that the system as a whole is a two phase one as defined by the phase rule. The protein solution, therefore, consists of one phase. The solubility experiments with pepsin (24) and trypsin give the same result. These results show that the catalytic reactions caused by pepsin and trypsin in protein solutions are homogeneous rather than heterogeneous.

### SUMMARY

A method is described for isolating a crystalline protein of high tryptic activity from beef pancreas. The protein has constant proteolytic activity and optical activity under various conditions and no indication of further fractionation could be obtained. The loss in activity corresponds to the decrease in native protein when the protein is denatured by heat, digested by pepsin, or hydrolyzed in dilute alkali.

The enzyme digests casein, gelatin, edestin, and denatured hemoglobin, but not native hemoglobin. It accelerates the coagulation of blood but has little effect on the clotting of milk. It digests peptone prepared by the action of pepsin on casein, edestin or gelatin.

The extent of the digestion of gelatin caused by this enzyme is the same as that caused by crystalline pepsin and is approximately equivalent to tripling the number of carboxyl groups present in the solution.

The activity of the preparation is not increased by enterokinase.

The molecular weight by osmotic pressure measure is about 34,000.

The diffusion coefficient in  $\frac{1}{2}$  saturated magnesium sulfate at 6°C. is  $0.020 \pm 0.001$  cm.<sup>2</sup> per day, corresponding to a molecular radius of  $2.6 \times 10^{-7}$  cm.

The isoelectric point is probably between pH 7.0 and pH 8.0.

The optimum pH for the digestion of casein is from 8.0–9.0.

The optimum stability is at pH 1.8.

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## CRYSTALLINE TRYPSIN

### III. EXPERIMENTAL PROCEDURE AND METHODS OF MEASURING ACTIVITY

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A number of methods may be used to follow the digestion of proteins by trypsin (1). Since we were primarily interested in the protein-splitting enzyme, the methods for measuring the activity were chosen so as to be limited as much as possible to the changes in the protein itself rather than the later steps in the digestion. This is all the more necessary in the case of trypsin since it is known that digestion of proteins by crude pancreatic extract consists of a series of reactions. The first detectable change in protein solutions caused by proteolytic enzymes is a marked change in viscosity of the solution. This is followed by the appearance of nitrogen in a form not precipitated by trichloroacetic acid and also by an increase in the number of carboxyl and amino groups. The change in viscosity is probably entirely due to changes in the protein itself and so measures only the hydrolysis of the protein. The formation of nitrogen not precipitated by trichloroacetic acid also measures principally changes in the protein but also some of the subsequent changes. The increase in carboxyl groups measures the total hydrolysis and, as usually carried out, does not measure the hydrolysis of the protein itself.

This is owing to the very high molecular weight of the protein. In the case of casein, for example, 5 ml. of 5 per cent casein at pH 7.0 gives a formol titration of about 9 ml. N/50 sodium hydroxide. It is therefore about 0.04 normal. Since the molecular weight of casein is about 100,000 (2), a 5 per cent solution is  $\frac{50}{1 \times 10^5} = 0.0005$  molar. Therefore, each molecule must contain about 100 titrable carboxyl groups. If all the casein molecules were split once there would result an increase of only one more group and the titration would increase  $\frac{1}{100} \times 9 = 0.09$



ml. N/50 alkali, which is close to the error of titration. Complete destruction of the casein could therefore occur before any increase in titration could be accurately determined even with N/50 alkali. This fact has led to the statement (3) that there is a purely physical change in protein solution not accompanied by any chemical change. It follows, on the other hand, that an increase in titration of 9 ml. N/50 sodium hydroxide (equivalent to 0.9 ml. N/5) must represent the appearance of 100 new carboxyl (or amino) groups. It is evident, therefore, that the increase of formol titration, if carried out with N/5 alkali measures principally the later stages of digestion and even if carried out with N/50 alkali can hardly be considered as the result of hydrolysis of the protein itself.

Any method of expressing enzymatic activity, to be significant, must yield figures which are independent of the concentration of the enzyme solution used. In general the amount of change caused by the enzyme is only proportional to the enzyme concentration in the early stages of the reaction and for this reason, in addition to the relation to the protein, the determination must be restricted to the initial stages. The activity units used in the present work, therefore, are defined in terms of the initial slope of the reaction curve. The determination is confined to that portion of the digestion curve which is a straight line, within the experimental error.

Evidence has frequently been presented to show that there are several proteolytic enzymes in pancreatic extract (4) which differ in their relative speed of hydrolysis of various proteins, especially gelatin. An enzyme especially active in the hydrolysis of gelatin was found to be present (5) in crude pepsin preparations. In order to detect such effects it is advisable to use various proteins. In the present work, therefore, the activity has been determined by the change in viscosity of gelatin and casein solutions, the increase in formol titration of gelatin solutions, and the increase in formol titration, and in the appearance of non-protein nitrogen in casein solutions.

The activity has been defined in terms of the reaction rate as follows (all reactions were carried out at 35.5°C.).

*Gelatin Viscosity* [T. U.]<sup>48V</sup>.—A change of 1 per cent per minute in the specific viscosity<sup>1</sup> of 2.5 per cent gelatin solution, containing 1/8 saturated ammonium sulfate and M/10 pH 4.0 acetate buffer.

<sup>1</sup> Specific viscosity is defined as the viscosity of the solution minus that of water; i.e.,  $\frac{\eta_s - \eta_{H_2O}}{\eta_{H_2O}}$ .

*Gelatin Formol* [T. U.]<sup>Gel. F</sup> or *Casein Formol* [T. U.]<sup>Cas. F</sup>.—Liberation of carboxyl (amino) groups from 6 ml. standard digestion mixture pH 7.6 in M/10 phosphate buffer pH 7.6 at the rate of 1 milliequivalent per minute.

*Casein, Soluble Nitrogen* [T. U.]<sup>Cas. S</sup>.—Formation of nitrogen soluble in 5 per cent trichloroacetic acid from 6 ml. standard digestion mixture at the rate of 1 milliequivalent per minute.

*Casein Viscosity* [T. U.]<sup>Cas. V</sup>.—Change of 1 per cent per minute in the specific viscosity of 5 per cent casein solution pH 7.6 in M/10 phosphate buffer pH 7.6.

The specific activity of the various preparations is then defined as the number of units of activity which would be observed when a quantity of the enzyme preparation containing 1 mg. protein nitrogen was present in 5.2 (viscosity method) or 6 ml. of the digestion mixture. The specific activity is abbreviated as [T. U.]<sub>mg. P.N.</sub><sup>4gV</sup>, etc.

#### *Relation to Willstätter's Activity Unit (6)*

Willstätter's unit of tryptic activity is defined as a titration of 1.0 ml. N/5 KOH required for 10 ml. 3 per cent casein solution after 20 minutes digestion at 30°C., which is equivalent approximately to 0.01 milliequivalent per minute per 10 ml. 3 per cent casein. This is about 1/100 of the unit used in this paper. Actually the activity of the purified fraction cannot be determined by Willstätter's method, since as discussed above, the form of curve is entirely different (7) from that obtained with crude preparations and the maximum increase, which it is possible to obtain under these conditions, is less than 1.0 ml. N/5 alkali.

#### *Preparation of Standard Protein Solutions*

*pH 4.0 Gelatin*.—100 ml. M/1 acetate buffer pH 4.0 added to 250 ml. saturated ammonium sulfate and made up with water to about 800 ml. 30 gm. air-dry isoelectric gelatin (8) (25 gm. by dry weight) added and the suspension allowed to stand at room temperature for about  $\frac{1}{2}$  hour, the volume made up to 1 liter and the gelatin dissolved by warming to 45°C., a few crystals of thymol added, the solution filtered through hard paper into a number of 150 ml. flasks, and stored in the ice box.

*pH 7.6 Gelatin*.—50 gm. of isoelectric gelatin added to 1 liter M/10 pH 7.6 phosphate buffer + 3 ml. 5 molar sodium hydroxide. The suspension is allowed to stand at room temperature for about  $\frac{1}{2}$  hour and the gelatin dissolved by warming to 45°C. Thymol is added and the solution stored in the ice box.

*Casein Solution.*—50 gm. of Kahlbaum's casein (according to Hammarsten) stirred into 1 liter M/10 pH 7.6 phosphate buffer + 7 ml. 5 molar sodium hydroxide. The suspension is stirred at room temperature until the casein dissolves. The flask is then immersed in boiling water for  $\frac{1}{2}$  hour. Thymol is added and the solution stored in the ice box.

*Technique of the Determination.*—In general the determinations were carried out in the same way as already described for the determination of pepsin (9) so that it is not necessary to repeat the detailed procedure.

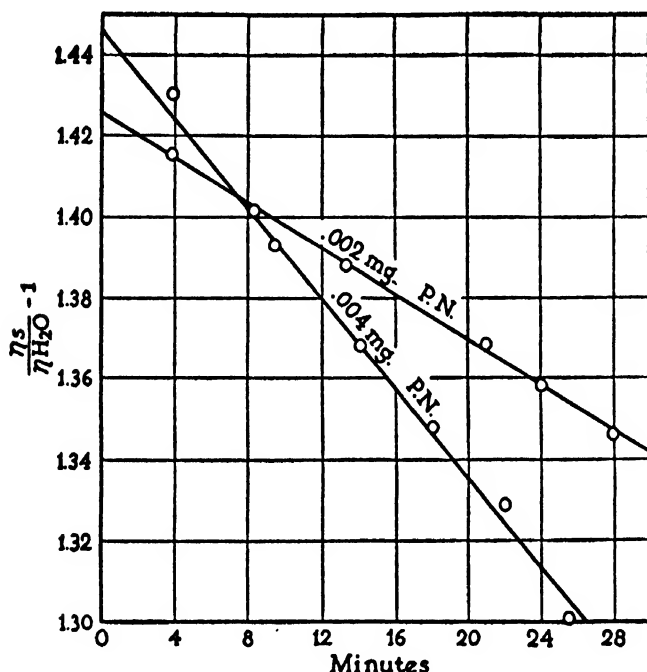


FIG. 1. Change in specific viscosity of 5.2 ml. standard gelatin solution with crystalline trypsin.

*Viscosity Determination.*—0.2 ml. of the enzyme solution in  $\frac{1}{2}$  saturated ammonium sulfate, made up in pH 4.0 acetate, or N/10,000 hydrochloric acid, is added to 5 ml. of the standard gelatin, or casein, solution previously warmed to 35.5°C., poured into the viscometer and the viscosity determined at short intervals. The viscosity is then plotted against the elapsed time and the per cent change in the specific viscosity per minute calculated from these curves. The curves obtained in this way with crystalline trypsin are shown in Fig. 1, and the calculation of the specific activity shown in Table I.

*Casein Non-Protein Nitrogen*—1 ml. of different dilutions of the enzyme solution are added to a series of tubes containing 5 ml. of the standard casein solution previously warmed to 35.5°C. and digestion allowed to proceed for 20 minutes. 5

ml. of 10 per cent trichloroacetic acid is then added. The suspension is filtered and the total nitrogen determined on 5 ml. of the filtrate. The curves determined in

TABLE I  
*Activity from Gelatin Viscosity*

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[T. U.] $\frac{4gV}{mg. P. N.}$ = per cent change in specific viscosity per min. per mg. trypsin nitrogen in 5.2 ml. gelatin	
	$= \frac{(1.445 - 1.39) \times 100}{1.45 \times 10 \times 4 \times 10^{-3}} = 95$
	or
	$\frac{(1.425 - 1.398) \times 100}{1.425 \times 10 \times 2 \times 10^{-3}} = 94$

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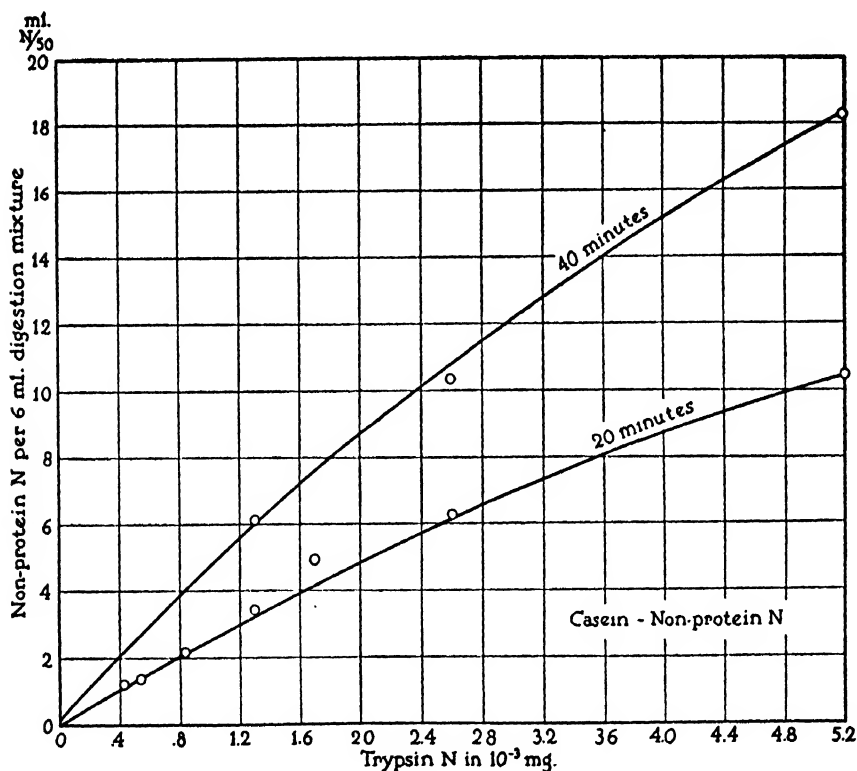


FIG. 2. Increase in soluble nitrogen in 6 ml. standard casein digestion mixture with crystalline trypsin.

this way with crystalline trypsin are shown in Fig. 2, and the calculation of the specific activity from the initial slope is shown in Table II. The value obtained in this way for the activity is independent of the time and also of the quantity of

enzyme used provided the observed change is within the initial portion of the curve which is nearly straight; *i.e.*, that part corresponding to a titration of less than 5 ml. *N*/50 alkali.

TABLE II  
*Activity from Casein Non-Protein Nitrogen*

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[T. U.]	Cas. S	= milliequivalents nitrogen soluble in 5
	mg. P. N.	per cent trichloracetic acid per min.
		per mg. trypsin nitrogen per 6 ml.
		digestion mixture
		$= \frac{2.5}{50 \times 20 \times 1 \times 10^{-3}} = 2.5$
		or
		$\frac{4.8 \times 1}{50 \times 40 \times 1 \times 10^{-3}} = 2.4$

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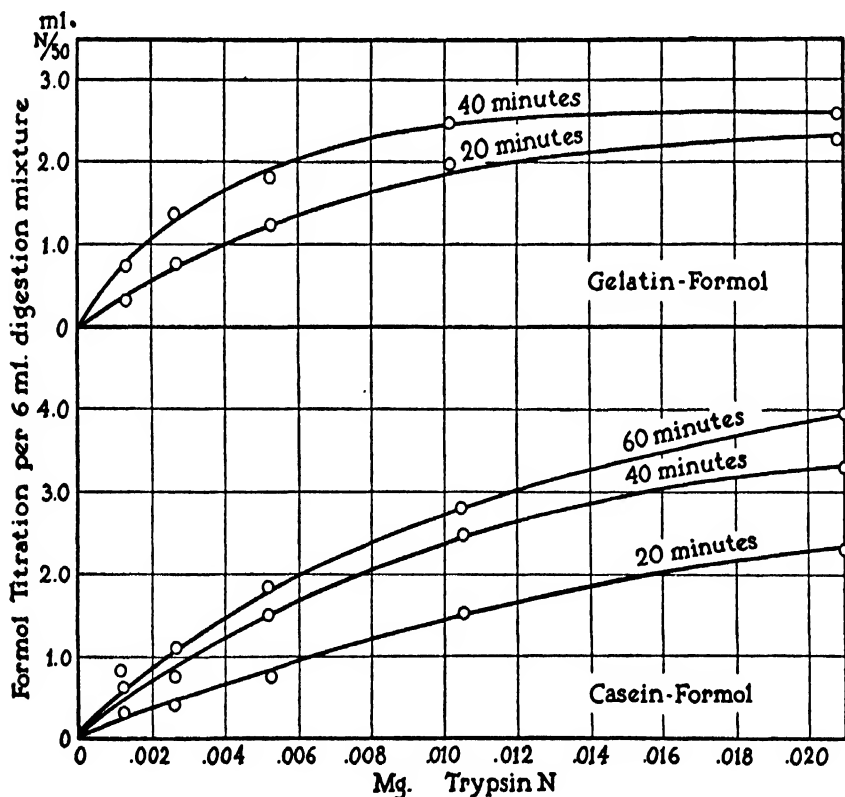


FIG. 3. Increase in formol titration in 6 ml. standard casein or gelatin digestion mixture with crystalline trypsin.

*Casein Formol or Gelatin Formol.*—These determinations are carried out in the same way as for non-protein nitrogen except that the entire solution is titrated with  $N/50$  alkali in the presence of formaldehyde, as described in the pepsin determinations.<sup>2</sup> Curves obtained with crystalline trypsin and casein are shown in Fig. 3, and the calculation of the specific activity from these curves in Table III. Curves obtained with crystalline trypsin and gelatin are shown in Fig. 3, and the calculation of the activity in Table IV. These curves were obtained by using different quantities of enzyme for the same time, but precisely the same curves could

TABLE III  
*Activity from Casein Formol*

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[T. U.] <sup>Cas. F</sup> mg. P. N. = milliequivalents carboxyl groups	
per min. per mg. trypsin nitro-	
gen per 6 ml. digestion mixture =	
	$\frac{0.5}{50 \times 20 \times 3 \times 10^{-3}} = 0.17$
	or
	$\frac{0.5}{50 \times 40 \times 1.4 \times 10^{-3}} = 0.18$
	or
	$\frac{0.5}{50 \times 60 \times 1.0 \times 10^{-3}} = 0.17$

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TABLE IV  
*Activity from Gelatin Formol*

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[T. U.] <sup>Gel. F</sup> mg. P. N. = milliequivalents carboxyl groups	
per min. per mg. trypsin nitro-	
gen per 6 ml. digestion mixture =	
	$\frac{0.5}{50 \times 20 \times 1.8 \times 10^{-3}} = 0.28$
	or
	$\frac{0.5}{50 \times 40 \times 0.8 \times 10^{-3}} = 0.31$

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be obtained by determining the amount of digestion with a constant quantity of enzyme for different times. This peculiarity is due to the fact that the amount of digestion is determined by the product of the enzyme concentration and the time, and a given value for this product represents a constant amount of digestion no matter how the enzyme concentration or time is varied provided the product remains constant.

<sup>2</sup> The titration when carried out in this way gives figures which are about 10 per cent lower than those obtained by Willstätter's alcohol method (Rona, p. 307).

*Rennet Action.*—The effect on the clotting of milk was determined precisely as described for pepsin (9).

*Effect of the Clotting of Blood.*—The method of Wohlgemuth as described by Rona (10) was used. It consists in determining the quantity of enzyme necessary to cause the coagulation of 3 ml. of a 1/10 dilution of magnesium sulfate plasma. The diluted plasma was brought to 0.02 molar calcium chloride as the enzyme was not active in the complete absence of calcium.

*Amylase Determination.*—Amylase determination was carried out essentially as described by Willstätter (11) except that instead of titration, the quantity of enzyme required to destroy the blue color with iodine was determined.

*Lipase Test.*—Olive oil was stirred with an equal volume of 10 per cent sodium carbonate solution and then washed in a separatory funnel with water. A sample of the emulsion gave a slight pink color with phenolphthalein. 1 ml. of enzyme solution, 0.8 ml. of glycerin, and 8 ml. of emulsion were mixed. 1 drop of phenolphthalein added and the emulsion titrated with  $N/10$  sodium hydroxide to color of a phenolphthalein standard. The suspension was then left at 30°C. for 3 days and titrated with  $N/10$  sodium hydroxide to a deep pink color.

*Erepsin.*—1 ml. of the enzyme solution was added to 5 ml.  $M/10$  pH 8.0 glycylglycine and the increase in titration determined after 24 hours.

*Enterokinase Solution.*—The enterokinase solution was prepared according to Waldschmidt-Leitz (12). The glycerin extract of dried pancreas was prepared according to Willstätter and Waldschmidt-Leitz (13).

*Determination of Protein Nitrogen.*—Since most of the solutions used in these experiments contained ammonium sulfate it was necessary to determine the protein nitrogen rather than total nitrogen. 5 ml. of the sample was mixed with 5 ml. of either 5 per cent or 20 per cent trichloroacetic acid, the mixture warmed to 70°C. for 10 or 15 minutes, and then allowed to cool to room temperature. The suspension was filtered through hardened paper (No. 575, S. and S. 5  $\frac{1}{2}$  cm.) and the precipitate washed with 2.5 or 10 per cent trichloroacetic acid until the filtrate gave no test for ammonia with Nessler's reagent. The precipitate was then washed with a fine stream of distilled water into Kjeldahl flasks and the nitrogen determined as usual.

*Micro Kjeldahl Determinations.*—Micro Kjeldahl determinations were done as previously described (14) except that 2 drops selenium oxychloride ( $SeOCl_2$ ) is used instead of the mercuric oxide, as suggested by Lauro (15). It is, therefore, not necessary to use the sodium hypophosphite solution.

The method of phosphorus determinations described by Sørensen (16) was used.

*Method of Solubility Determinations.*—The same general methods were used as already described in the case of pepsin (14).

The numerous analytical determinations were made by Mr. Nicholas Wuest and Miss Margaret R. McDonald.

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## CRYSTALLINE TRYPSIN

### IV. REVERSIBILITY OF THE INACTIVATION AND DENATURATION OF TRYPSIN BY HEAT

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It was noted by Mellanby and Wooley (1) that trypsin solutions in dilute acid could be heated nearly to boiling with very little loss in activity, and this observation was confirmed by Eddie (2). At temperatures below 40°C., on the other hand, the enzyme is more stable near pH 4 or 5 than it is in acid solution. This latter result has also been obtained by the writer (3) and by Pace (4). In the course of the preparation of crystalline trypsin by Kunitz and the writer (5) it was found that the further purification proceeded the more heat-stable the preparation became. The final crystalline material may be heated to boiling in dilute solution over the whole range of acidity between pH 1 and pH 7 with little or no loss in activity and apparently without the formation of any denatured protein. This behavior is remarkable, since in general heating denatures proteins and inactivates enzymes. The result is, however, somewhat analogous to that in the case of serum albumin. Spiegel-Adolf (6) found that serum albumin also could be heated in certain pH ranges without the appearance of any denatured protein in the solution after it had been cooled. Spiegel-Adolf, and Anson and Mirsky (7) also showed that the denaturation of serum albumin is easily reversible and it is probable that the fact that no denatured protein is found in the solution after cooling is due to the reformation of native from denatured protein on cooling. It has also been found in the case of crystalline pepsin (8) that the activity is lost when the protein is denatured and regained when the denaturation is reversed. The possibility therefore exists in the case of trypsin that inactivation of the enzyme and denaturation of the protein occur, as

would be expected, at higher temperatures; but that on cooling the denaturation is reversed and the native, active protein re-formed.

*Presence of Denatured Protein in Hot Trypsin Solutions.*—If the protein is actually present in the denatured form at high temperatures, it would be expected that rapid addition of the hot enzyme solution to cold salt solution would result in the precipitation of insoluble, denatured protein<sup>1</sup> while if the enzyme solution were allowed to cool, as is usually done, and then added to the cold salt solution, no denatured protein would be found. If the native protein only is active then it would also be expected that the filtrate from the denatured protein

TABLE I

*Effect of Repeated Heating and Cooling on the Activity and Solubility of Trypsin*

0.5 gm. trypsin dissolved in 25 ml. N/20 HCl heated to 70°C. and samples taken for total and soluble protein and activity. Solution cooled quickly to 20°C. and sampled in the same way; solution then reheated to 70°C. and another sample taken.

Temp.	Time	Total			Soluble		
		[P. N.] <sub>ml.</sub>	[T. U.] <sup>4gV</sup> <sub>ml.</sub>	[T. U.] <sup>4gV</sup> <sub>mg. P. N.</sub>	[P. N.] <sub>ml.</sub>	[T. U.] <sup>4gV</sup> <sub>ml.</sub>	[T. U.] <sup>4gV</sup> <sub>mg. P. N.</sub>
°C.	hrs.	mg.			mg.		
20	0.10	1.0	102	100	1.0	102	102
70	0.10	0.9	100	110	Trace	0.1	
20	0.10	1.0	108	108	1.0	105	105
70	0.10	1.1	108	106	Trace	0.1	

would be inactive while the solution of the enzyme which had been cooled would be active. This is actually the case as is shown in Table I.

This experiment shows that when a solution of trypsin in N/20 HCl at 70°C. is added to one-half saturated ammonium sulfate, without previous cooling, practically all of the protein precipitates and the supernatant liquid is inactive. If the enzyme solution is cooled before being added to the salt solution, on the other hand, no precipitate is formed and the solution contains its original activity. The heating

<sup>1</sup> Denatured protein is defined as that form of the protein which is insoluble at the isoelectric point or in the presence of strong salt solution but may be soluble in acid or alkali in the absence of salt.

and cooling may be repeated almost indefinitely without loss of activity provided the solution is not left at 70°C. for too long a time. This observation, in addition to the fact that there is no appreciable change in total protein nitrogen on heating shows that the absence of precipitable protein in the cooled solution is not due to digestion of the protein. The experiment confirms the idea that the protein is denatured in the hot solution but reverts to the native condition on cooling.

*Effect of Temperature on Rate of Reversal.*—The experiment may be varied by cooling the enzyme solution very rapidly to zero degree before adding it to the salt solution. It might be expected by analogy with the results with hemoglobin (Mirsky and Anson (9)) that the re-

TABLE II

*Reformation of Soluble Protein at 0° after Boiling in Acid*

0.1 gm. trypsin dissolved in 5 ml. N/20 HCl heated rapidly to boiling and poured into 50 ml. of a mixture of N/20 HCl and finely cracked ice. Samples taken at times and temperatures noted and soluble activity determined.

Temperature, °C. ....	20	100	0	0	0
Time, sec. ....	0	5	10	30	600
Soluble [T. U.] <sup>4gV</sup> ml. ....	4	0	1	1.4	3.7
Insoluble protein. ....	—	++++	+++	++	—

versal would take place slowly enough at zero degree to be measured. This actually is the case as is shown in Table II. The enzyme protein when cooled very rapidly to zero degree is still in the denatured form and precipitates at first completely with salt. As the solution stands at zero degree the denatured protein changes to the native form and at the same time the activity begins to reappear in the supernatant solution so that after about 10 minutes all the protein has reverted to the native form and the activity is again all found in solution. This experiment rules out any secondary reaction which might be supposed to occur when the hot enzyme solution is added to cold salt solution.

*Effect of Temperature on Equilibrium between Native and Denatured Protein.*—The preceding experiments show that there is a very rapid change from native to denatured protein at temperatures between zero and 90°C. They indicate that the temperature determines not

only the rate of transformation from native and active to denatured and inactive protein but also the equilibrium between the two forms. In order to determine whether or not this is really the case a trypsin solution was heated rapidly from 20° to 60°C. and then cooled again to 20°C. Samples were taken and added without previous cooling to cold salt solution at each 10° temperature interval and the soluble protein and activity determined. The result of this experiment is shown in Table III.

As the temperature rises the quantity of protein soluble in one-quarter saturated ammonium sulfate decreases rapidly and at 60°C. there is too little to determine. When the solution cools, soluble protein again

TABLE III

*Effect of Temperature on Equilibrium between Native and Denatured Protein*

0.6 gm. trypsin dissolved in 15 ml. N/20 HCl (pH of solution about 2). Stirred in tube in boiling H<sub>2</sub>O. 1 ml. samples taken at time and temperature noted and soluble nitrogen and activity determined. The tube is then put in cold water and samples taken as tube cools.

Time, min. ....	0		1		2		5		6
Temperature, °C. ....	20	40	50	60	80	60	50	40	20
[P. N.] <sub>ml.</sub> , mg. ....	0.22	0.16	0.07				0.054	0.12	0.15
[T. U.] <sub>ml.</sub> <sup>4gV</sup> ....	28	19	6.1	<1	<1	0.6	3.2	10	15
[T. U.] <sub>mg.</sub> P.N. ....	125	120	85				65	100	100

appears and the quantity present when cooled to 40°C. is nearly the same as that at 40°C. when the temperature was raised. The activity of the solution increases and decreases almost exactly in proportion to the quantity of soluble protein present. The experiment shows that equilibrium between native and denatured protein is very rapidly established and is extremely sensitive to changes in temperature so that at 20°C. the protein is present almost entirely as native protein while at 60°C. it is practically entirely denatured and inactive. It follows that the usual assumption that raising the temperature merely increases the rate of transformation from native to denatured, is not correct in this instance but that the temperature affects the equilibrium as well as the rate of the reaction. This possibility has been con-

sidered by Anson and Mirsky (10). Somewhat similar results have been noted with other enzymes in that the activity of a solution inactivated by heat has been found to increase on standing at lower temperatures. This result has been described by Bach and Wilenskii (11) in the case of purified peroxidase and has also been noted by Kulikoff and Bobkova (12) with trypsin and by Anson (13) with pepsin.

*Inactivation at Different Temperatures and pH 7.0.*—The preceding experiments show directly that the trypsin protein at temperatures above 50°C. is in a form which is insoluble in one-quarter saturated ammonium sulfate, that is to say, it is denatured. They also show directly that the specific activity of that part of the protein which is still native is the same as that of the original preparation. It is not possible to prove from these experiments, however, that the denatured protein is also inactive since, if the precipitate of denatured protein is added to protein solutions the precipitate dissolves and digestion occurs. It can be objected, therefore, that the apparent inactivity of the denatured protein precipitate is simply due to the fact that it is insoluble. It is necessary to show that the enzyme, while still in solution, is inactive at higher temperatures; but this cannot be done in  $N/15$  HCl since trypsin is not active under these conditions at any temperature. The experiments were, therefore, repeated at pH 7, under which conditions the enzyme is active at ordinary temperatures. It was found that the loss in activity when heated to 90°C. at pH 7 was a very sensitive test for the purity of the preparation. Preparations which contain relatively small amounts of inactive protein precipitate when heated at pH 7 and the activity does not return upon cooling. Carefully purified preparations may be heated in solution containing less than 1 mg. protein nitrogen per ml. and less than 0.05 molar salt to 90°C. at pH 7 for 5 to 10 minutes and cooled to 20°C. without any appreciable loss in activity. All preparations tested so far precipitate when heated at pH 7.5 to 9.5 and the activity is not recovered on cooling. Also, solutions which have been heated at pH 1 to 7 remain inactive if added before cooling to pH 8 to 10 buffer solutions. It is necessary at pH 7.0 to add the enzyme solution to the hot phosphate solution rather than to heat the mixture, since as will be seen later, the inactivation at intermediate temperatures of 50–70°C. is not reversible. There is at present no explanation for this peculiar fact, although it

was found that the denatured protein formed at 62°C. was different from that formed at 90°C.

In the following experiments, therefore, 1 ml. of the enzyme solution containing about 5 mg. protein nitrogen was added to 25 ml. of M/20 phosphate solution at the desired temperature. 1 ml. samples were taken at intervals of about 1 minute, added to 10 ml. cold N/10 HCl and this solution analyzed for total activity and total protein nitrogen. Another set of samples was taken and added at once, without cooling, to 5 ml. half saturated ammonium sulfate in M/20 pH 4 acetate buffer. These suspensions were then centrifuged and the supernatant liquid analyzed for activity and for protein nitrogen. This is called soluble activity or soluble protein nitrogen and, presumably, represents the amount of native protein in the heated solution.

*Changes in Soluble Protein or Activity.*—The results of these experiments are shown in the lower half of Fig. 1. The concentration of total protein, total activity, soluble protein, and soluble activity has been plotted as the  $\log_{10}$  of the per cent of the original concentrations. In the unheated solution all the protein and all the activity were present in the soluble form; *i.e.*, no precipitate was obtained when the solution was added to the ammonium sulfate. The higher the temperature the more rapidly the soluble protein or activity disappears until when the temperature is about 50°C. the process becomes so rapid as to render it impossible to make accurate measurements. At any one temperature the percentage loss of soluble protein is nearly the same as the percentage loss in soluble activity, as shown by the fact that the curves for the percentage of those quantities remaining in solution are parallel. In other words, the specific activity of the soluble (native) protein remains practically constant. This agrees with the idea that the denatured protein is inactive and that the activity is a property of the native protein molecule.

The experiments are not sufficiently accurate to permit calculation of the exact temperature coefficient, but it is more than two for an interval of 10° and therefore agrees qualitatively with the value usually found for the denaturation of proteins or the inactivation of enzymes. The course of the reaction, as plotted in the figure, is not monomolecular; since the reaction does not go to complete inactivation but stops at an intermediate value, depending on the temperature, the mono-

molecular constants should be calculated using the concentration of soluble protein or activity present at this equilibrium point rather

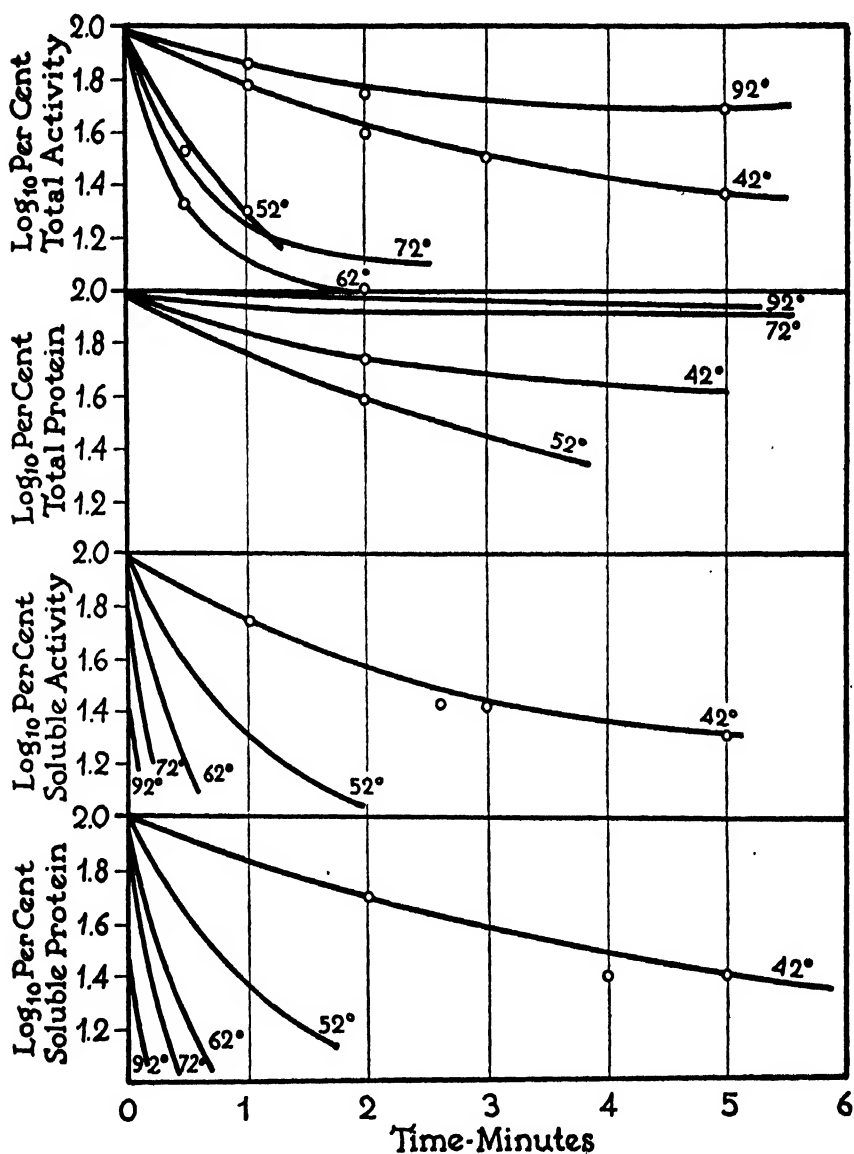


FIG. 1. Decrease in total activity, total protein, soluble activity, and soluble protein concentrations of trypsin solutions at pH 7.0 and various temperatures.

than the total amount present originally, as has been done in the figure. If the reaction velocity is calculated using this equilibrium value it is



found to be monomolecular within the wide error of the present experiment.

*Changes in Total Protein and Total Activity.*—The effect of increasing the temperature on the concentration of total protein or total activity, shown in the upper two series of curves, is entirely different from that on the concentration of soluble protein or activity discussed above. At 42°C. the quantity of total protein decreases with time but more slowly than does the soluble protein. At 52°C. it decreases faster than at 42°C. but at 72°C. or 92°C. it decreases much less rapidly than at 52°C. The rate of destruction of the total protein, therefore, goes through a maximum at about 52°C. and then becomes slower. The loss in total activity is nearly parallel to the loss in total protein except that in this case there is very rapid loss at 72°C. At 92°C. however, the activity is destroyed less rapidly than at 42°C. so that there is a general agreement between the decrease in total protein and the decrease in total activity. These results are in qualitative agreement with the idea expressed above, that the denatured, inactive protein found at temperatures above 40°C. reverts to active, native protein when the solution is cooled to 20°C. Since there is a decrease in total protein at 40–60°C., it is necessary to assume in addition that, at this pH, there is some digestion of the denatured protein by the native, active protein. At 42°C., for instance, 10 to 20 per cent of the protein is in the native, active form and, therefore, digests and destroys the denatured form present. The total activity, total protein, soluble activity, and soluble protein, therefore, all decrease.

At 52–62°C. more of the protein is changed rapidly to the denatured, inactive form but the rate of digestion is also increased by the increase in temperature so that this denatured protein is still digested by the small amount of native, active protein present and the total protein and total activity, therefore, still decrease with time. At the higher temperatures the protein is completely and rapidly changed to the denatured form and therefore no digestion occurs since no native, active protein is present. The total protein, therefore, remains constant and reverts to native protein when cooled so that there is no change in the total protein or total activity. The slow decrease in the total activity at 92°C. is probably due to a secondary change occurring in the denatured protein which is not reversible (14)

since all the work on the reversibility of protein denaturation shows that with prolonged heating a form is obtained which does not revert to the native condition. The loss in total activity at 62°C. and 72°C. without corresponding loss in total protein shows that the protein when heated at these temperatures does not regain its activity on cooling, although it does become soluble again.

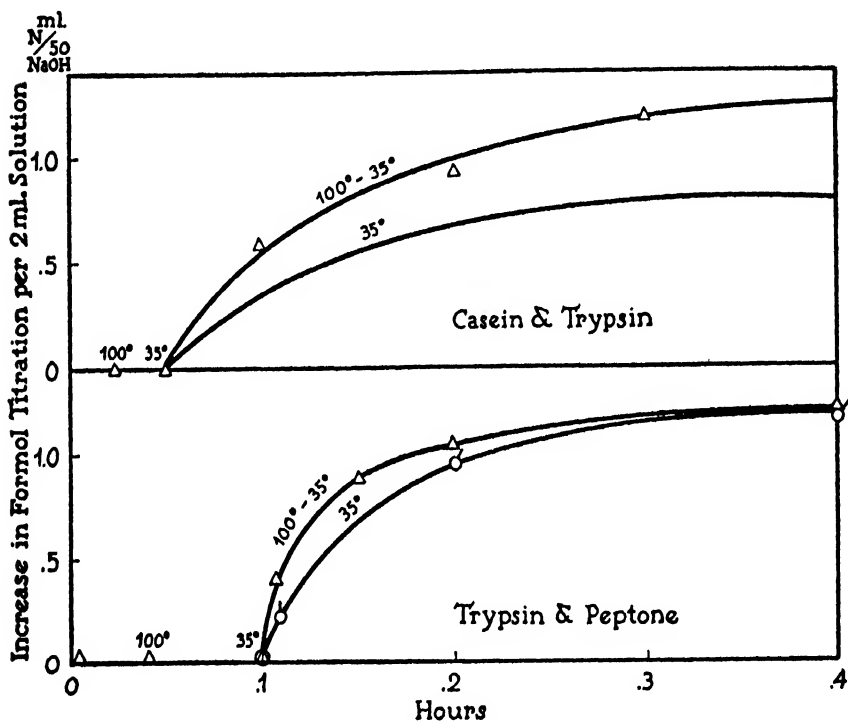


FIG. 2. Rate of digestion of casein or peptone solutions with trypsin at 100°C. and 35°C.

*Trypsin Is Inactive at 100°C.*—It was assumed in accounting for the fact that there was no loss in total protein at 92°C. that the enzyme was inactive at this temperature although, as the experiment shows, it was active in the solution after it had been allowed to cool. This assumption may be directly verified by determining the digestion of casein or of peptone at 100°C. and at 35°C. A solution of casein, or of peptone,<sup>2</sup> at pH 7 and 100°C. is not digested by trypsin. If the same solution is

<sup>2</sup> The rate of hydrolysis of different commercial peptone preparations with the crystalline trypsin varies greatly.

cooled to 35°C. rapid digestion occurs at once. The results of such an experiment are shown in Fig. 2, in which the increase in formol titration in 2 cc. of the digestion mixture has been plotted against the time. Two solutions of casein and of peptone were prepared; one of each was placed at 35°C. and the trypsin added; the other pair was heated to 100°C. and the same quantity of trypsin added. Samples were taken for formol titration at short intervals from all four solutions and after about 5 minutes the solutions at 100°C. were cooled rapidly to 35°C. The results show that no digestion occurred while the solutions were at 100°C. but that as soon as they were cooled to 35°C. rapid digestion commenced and proceeded at the same, or even greater rate,<sup>3</sup> than in the control solutions which had not been heated to 100°C. The curves have been plotted so that the time of adding the trypsin to the 35°C. solutions coincides with the time at which the 100°C. solutions were cooled to 35°C.

These experiments show directly that casein and peptone are not acted on by trypsin at 100°C. but are rapidly digested at 35°C. They indicate that the enzyme is inactive at 100°C. but it could be assumed that some reversible change occurs in the substrate at this temperature which renders it resistant to digestion by the enzyme. This appears unlikely and in addition it may be shown directly that the effect is on the enzyme rather than on the substrate by following the digestion of peptone solutions at zero degree upon the addition of hot or cold trypsin solutions.

It was shown before that trypsin solutions heated to 100°C. and cooled very rapidly to zero degree remained in the denatured form for some time as shown by the formation of a precipitate on the addition of ammonium sulfate. If trypsin solution at 100°C. is added rapidly to peptone solution at zero degree, it would be expected that digestion would occur much more slowly than if unheated trypsin were added, or than if the heated trypsin were allowed to cool slowly before being added to the peptone. The result of the experiment done in this way

<sup>3</sup> If the experiment is done with gelatin, however, no digestion occurs even after cooling the solution. The gelatin evidently prevents the reactivation of the enzyme and it is possible that some of the split products of trypsin itself act in the same way and that this is the explanation of the failure of the trypsin to reactivate after being heated to 72°C.

is shown in Fig. 3. The experiment shows that the peptone is digested at about the same rate by the trypsin solution before heating or, if it is allowed to cool slowly after heating, but if the hot trypsin solution is added at once to the cold peptone the rate of digestion is much slower. This experiment confirms the assumption made before that the enzyme is inactive at 100°C. and that the failure to digest casein at 100°C. is

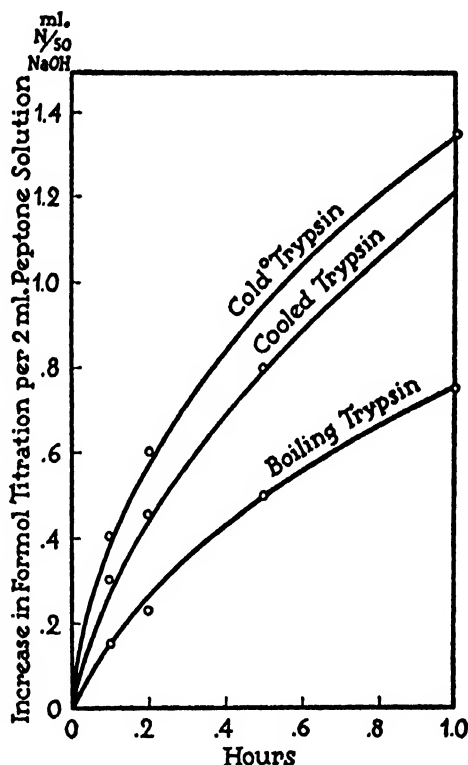


FIG. 3. Rate of digestion of peptone solutions on the addition of cold, hot, and cooled trypsin solutions.

due to the fact that the enzyme is in an inactive form at this temperature rather than that the casein or peptone is in a resistant form.

*Determination of S-S Groups in the Insoluble Protein.*—The conclusion that the insoluble, denatured protein is inactive is further confirmed by determination of the S-S groups in the precipitate formed by the addition of hot trypsin solutions to salt solutions. Mirsky and Anson (15) have shown that denatured proteins differ from native protein in that the S-S groups are free in the denatured form. The writer

is indebted to Drs. Mirsky and Anson for an analysis of the insoluble precipitate obtained by adding hot trypsin solution to cold salt. The analysis showed that the protein contained all the S-S groups in the free form and, therefore, agrees in this respect with other denatured proteins.

*Formation of Native, Active Protein from Denatured Protein.*—The experiments already described show that trypsin at a temperature of 90°C. is inactive and that the trypsin protein precipitates when added to one-quarter saturated ammonium sulfate. This precipitate of denatured protein may be partially reconverted to native, active protein by dissolving in N/50 hydrochloric acid. The precipitate dis-

TABLE IV  
*Reactivation—Precipitated, Denatured Trypsin*

	No.	Vol.	[T. U.] <sub>ml.</sub> <sup>4gV</sup>	[T. U.] <sub>total</sub> <sup>4gV</sup>
0.5 gm. trypsin cake dissolved in 15 ml. H <sub>2</sub> O. Solution 1 poured into 70 ml. boiling pH 7.0 M/10 PO <sub>4</sub> , poured into 80 ml. 0.7 saturated ammonium sulfate M/10 pH 4.0 acetate, centrifuge supernatant. . . . .	1	15	200	3000
Precipitate + 75 ml. N/50 HCl, stir for 2 hrs. at 35°C. . . . .		165	0	0
		75	14	1050

solves slowly and the solution then contains from 30 to 40 per cent of the original activity. It also contains a soluble protein which is inactive. This soluble, inactive protein is formed from the active trypsin protein on standing at room temperature in dilute acid solution. This reaction was discussed in connection with the preparation of the enzyme (5). The result of this experiment is shown in Table IV.

#### DISCUSSION

Trypsin solutions which have been heated to boiling and then cooled show no loss in activity or formation of denatured protein. The preceding experiments show that these anomalous results are due to the existence of an equilibrium between native, active protein and denatured, inactive protein. Below 30°C. the protein is nearly all in the active and native condition while above 65°C. it is practically all

denatured and inactive. Equilibrium is reached rapidly from either side. The loss in activity is proportional to the formation of denatured protein and when the denatured protein changes back to the native condition the activity is also recovered. This is good evidence that the activity of the preparation is a property of the native protein molecule since, if it be assumed that the activity is due to a special active molecule associated with the protein, it is further necessary to suppose that the conditions for inactivating and reactivating this hypothetical active molecule are quantitatively the same as those for the formation of denatured from native and native from denatured protein.

They agree, therefore, with the results with pepsin denatured by alkali or by heat and it is probable that the same mechanism accounts for the recovery of the activity of other enzyme solutions after heating, recorded in the literature.

They are also very similar to the result with hemoglobin, since this protein when denatured also loses its characteristic property, that of reversible combination with oxygen and recovers this property when the denaturation is reversed (16).

### *Experimental Procedure*

*Trypsin.*—The trypsin preparation used was crystallized from about 0.7 saturated ammonium sulfate, as described by Northrop and Kunitz (5). It was kept in the form of a moist filter cake at 5°C. The figures given for the weight of trypsin refer to the weight of this filter cake.

*Activity Units* (T.U.)<sup>4gV</sup>—The activity is expressed as per cent change per minute in the viscosity of gelatin solutions. The determination was carried out as previously described (17).

*Gelatin.*—Gelatin was prepared from Cooper's powdered gelatin as described by Northrop and Kunitz (18).

*Casein.*—Casein solutions were made up from Kahlbaum's casein "according to Hammarsten."

*Peptone.*—The preparation used was a sample of Fairchild's peptone which contained more protein nitrogen than usual. Most samples of commercial peptone tried were not digested by the trypsin.

*Total Activity.*—1 ml. of trypsin solution added to 10 ml. of N/20 hydrochloric acid and allowed to stand at 20°C. for about  $\frac{1}{2}$  hour and analyzed for activity with standard gelatin solution as usual.

*Total Protein Nitrogen.*—A volume of solution containing about 1 mg. protein nitrogen is added to an equal volume of 5 per cent trichloroacetic acid and warmed

to 70°C. for 5 minutes. The suspension is then cooled, filtered through hardened paper, and the precipitate washed with 2½ per cent trichloroacetic acid until the washing gives no test for ammonia. The precipitate is then washed into a Kjeldahl flask and analyzed for total nitrogen.

*Soluble Activity and Soluble Protein Nitrogen.*—1 ml. of solution is added to 5 ml. half-saturated ammonium sulfate in N/20 pH 4 acetate buffer. The suspension is centrifuged and the supernatant solution analyzed for activity and protein nitrogen as described above.

The analytical determinations were done by Mr. N. Wuest.

#### SUMMARY

1. If dilute solutions of purified trypsin of low salt concentration at pH from 1 to 7 are heated to 100°C. for 1 to 5 minutes and then cooled to 20°C. there is no loss of activity or formation of denatured protein. If the hot trypsin solution is added directly to cold salt solution, on the other hand, all the protein precipitates and the supernatant solution is inactive.

2. The per cent of the total protein and activity present in the soluble form decreases from 100 per cent to zero as the temperature is raised from 20°C. to 60°C. and increases again from zero to 100 per cent as the solution is cooled from 60°C. to 20°C. The per cent of the total protein present in the soluble (native) form at any one temperature is nearly the same whether the temperature is reached from above or below.

3. If trypsin solutions at pH 7 are heated for increasing lengths of time at various temperatures and analyzed for total activity and total protein nitrogen after cooling, and for soluble activity and soluble (native) protein nitrogen, it is found that the soluble activity and soluble protein nitrogen decrease more and more rapidly as the temperature is raised, in agreement with the usual effects of temperature on the denaturation of protein. The total protein and total activity, on the other hand, decrease more and more rapidly up to about 70°C. but as the temperature is raised above this there is less rapid change in the total protein or total activity and at 92°C. the solutions are much more stable than at 42°C.

4. Casein and peptone are not digested by trypsin at 100°C. but when this digestion mixture is cooled to 35°C. rapid digestion occurs.

A solution of trypsin at 100°C. added to peptone solution at zero degree digests the peptone much less rapidly than it does if the trypsin solution is allowed to cool slowly before adding it to the peptone solution.

5. The precipitate of insoluble protein obtained from adding hot trypsin solutions to cold salt solutions contains the S-S groups in free form as is usual for denatured protein.

6. The results show that there is an equilibrium between native and denatured trypsin protein the extent of which is determined by the temperature. Above 60°C. the protein is in the denatured and inactive form and below 20°C. it is in the native and active form. The equilibrium is attained rapidly. The results also show that the formation of denatured protein is proportional to the loss in activity and that the re-formation of native protein is proportional to the recovery of activity of the enzyme. This is strong evidence for the conclusion that the proteolytic activity of the preparation is a property of the native protein molecule.

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## CRYSTALLINE TRYPSIN

### V. KINETICS OF THE DIGESTION OF PROTEINS WITH CRUDE AND CRYSTALLINE TRYPSIN

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The kinetics of trypsin digestion have been studied by several workers (1-8). The results are anomalous from the point of view of the theory of simple catalysis both as regards the effect of the concentration of substrate and the time course of the reaction. These studies have been made with crude pancreatic extracts which undoubtedly contain several enzymes and some of the anomalous results may be due to this fact. The crystalline trypsin prepared by Kunitz and the writer (9) appears to be a chemical individual and certainly contains fewer enzymes than crude pancreas extract. The kinetics of the digestion of gelatin, casein, and hemoglobin with crude pancreatic extract and crystalline trypsin have been studied. The results of these experiments are contained in this paper.

#### *Extent of the Reaction*

The crystalline trypsin increases the formol titration of casein by about 100 per cent, equivalent to about 100 hydrolyses per mole, and the formol titration of gelatin solutions by about 200 per cent, equivalent to about 60 hydrolyses per mole. The crude preparations cause about three times as much hydrolysis as does the crystalline (9).

#### *Effect of the Concentration of Substrate*

The rate of digestion of various concentrations of gelatin, casein, and hemoglobin with crude or crystalline trypsin were determined at 35°C. The amount of digestion was followed by the increase in formol titration with casein and hemoglobin and also by decrease in

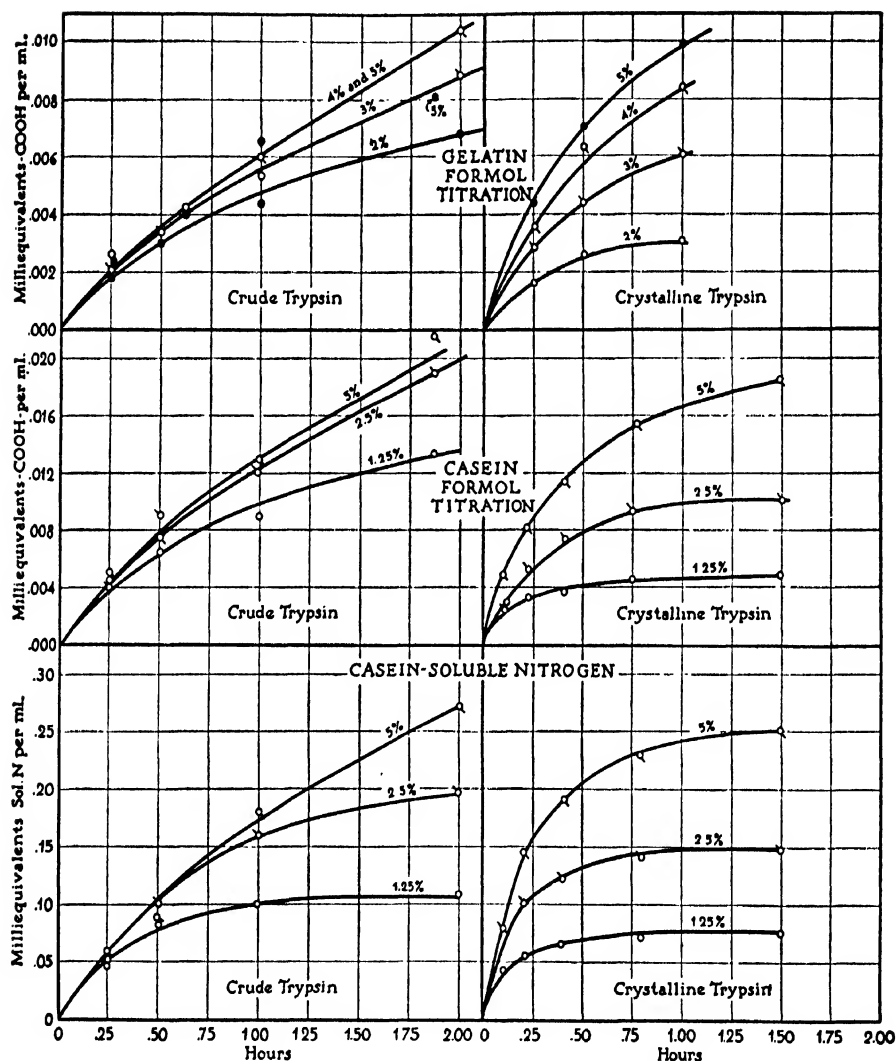


FIG. 1. Digestion of various concentrations of gelatin and casein with crude and crystalline trypsin.

protein soluble in 2.5 per cent trichloroacetic acid. The results of these experiments are shown in Figs. 1 and 2. The experiments show that for the first 30–40 per cent of the reaction the amount of digestion with crude trypsin is the same for 2.5 and 5 per cent protein concentration. In other words, the amount of digestion instead of being proportional to the substrate concentration, as predicted by the simple theory, becomes independent of it. This result is frequently observed with enzymes and has usually been ascribed to the formation of an intermediate compound. With the crystalline trypsin this anomalous

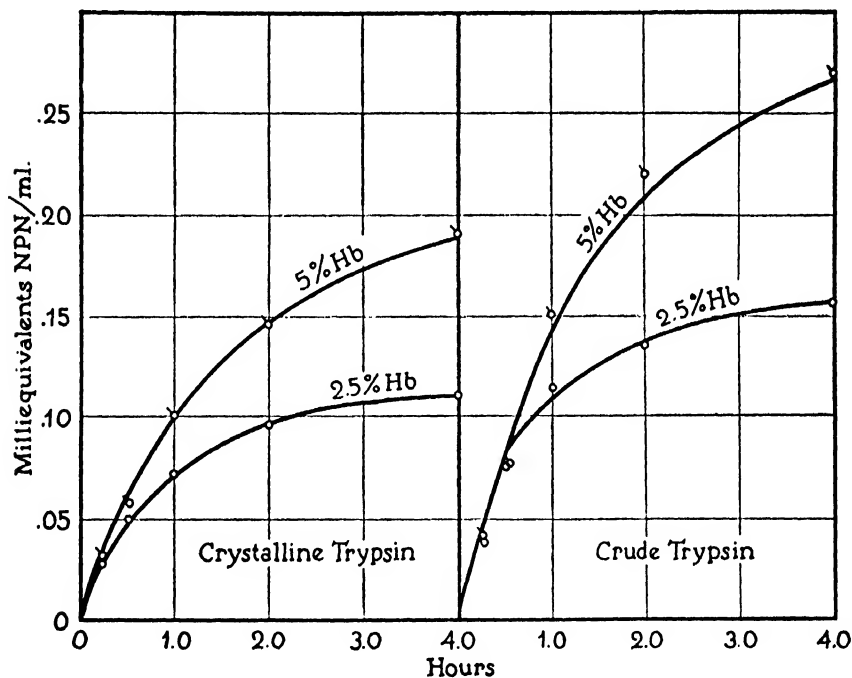


FIG. 2. Digestion of hemoglobin with crude and crystalline trypsin

result is much less marked, the amount of digestion being nearly that expected from the change in the substrate concentration. The first few per cent of the reaction still show this effect, however, especially in the case of casein and hemoglobin when the digestion is followed by means of the change in protein nitrogen.

#### *Kinetics of the Reaction*

The digestion of casein as determined by the formation of non-protein nitrogen with the crude trypsin preparation follows the course

of a monomolecular reaction quite closely although the value of the velocity constant is nearly inversely proportional to the protein concentration instead of being independent of this value. This is the result previously obtained (7, 9, 13). With crystalline trypsin the rate of digestion decreases more rapidly than with the crude trypsin and the velocity constants drop quite rapidly as the reaction proceeds. This agrees with the results of Schönfeld-Reiner (11). This is more marked with dilute protein than with concentrated so that with crystalline trypsin the value of the constants for the two protein concentrations approach each other quite rapidly. These results are shown in Table I. The values for the velocity constant have been tabulated at corresponding total amounts of digestion so that they are

TABLE I

*Kinetics of Hydrolysis of 2.5 and 5 Per Cent Casein with Crude and Crystalline Trypsin*

$$K = 1/T \log_{10} A_t/(A_t - A)$$

A	Crude trypsin with		Crystalline trypsin with	
	2.5 per cent casein	5 per cent casein	2.5 per cent casein	5 per cent casein
	K	K	K	K
0				
2.0	0.50	0.21	0.084	0.04
4.0	0.54	0.22	0.080	0.04
6.0	0.55	0.22	0.050	0.03
8.0	0.57	0.23	0.030	0.03
10.0	0.50	0.21	0.026	0.026

$A_t$  for 2.5 per cent = 11.0 = final total amount.

for 5.0 per cent = 22.0 = " " "

comparable. This result might be due to the fact that casein is a mixture or solid solution of several different proteins (12). The experiment was therefore repeated with hemoglobin, which is probably a pure substance. The reaction shows the same abnormal behavior with hemoglobin. In this case the monomolecular constants drop quite rapidly with both purified and crude trypsin.

#### *The Rate of Digestion of Mixtures of Casein and Gelatin*

The writer has pointed out before (2) that although the effect of changing the substrate concentration agrees with the hypothesis of

intermediate compounds, the results of experiments in which the rate of digestion of a mixture of proteins is followed, do not agree with this hypothesis. If the fact that a 5 per cent gelatin or casein solution digests at the same rate as 2.5 per cent be due to the saturation of the enzyme with the protein, then the addition of casein to a 5 per cent gelatin solution should cause no increase in the rate of digestion since it has already been assumed that the enzyme is saturated with the gelatin. If the rate of digestion of the casein alone is determined in such a mixture, then it would be predicted that the digestion of the casein in the presence of the gelatin would be much slower than the rate of digestion of the same concentration of casein alone. On the other hand, if no intermediate compound is formed then it would be expected that the rate of digestion of a mixture of casein and gelatin would be equal to the sum of the rates of the two proteins separately. In determining the rate, however, it is necessary, owing to the inhibitory effects of the products of the reaction, to compare the curves at a point of equal total digestion rather than at equal time intervals. The quantity of protein digested after a given time interval would not be expected to be equal to the sum of the two quantities separately. This point has been overlooked by Westenbrink (10) who has erroneously quoted the writer as predicting that the amount of digestion of a mixture at a given time would be equal to the sum of the amounts digested in the two solutions separately. Westenbrink's results even when recalculated, however, show less difference between the rate of digestion of mixtures of casein and gelatin and of gelatin alone than the writer's experiments, so that the experiments have been repeated. The results of experiments in which the digestion of mixtures of 5 per cent casein and 5 per cent gelatin, are compared to the rates of digestion of the two proteins separately, are shown in Fig. 3. They confirm the earlier experiments of the writer (2) in that the rate of digestion of the mixture is greater than the rate of digestion of either protein alone and is nearly equal to the sums of the rates of the two proteins alone, especially in the case of the crystalline trypsin. The lower part of Fig. 3 shows that 5 per cent casein in the presence of 5 per cent gelatin digests at identically the same rate as does 5 per cent casein alone. As stated before (3), this result is difficult to account for on the basis of an intermediate compound unless it be further assumed that there are two enzymes, one for each protein. It cannot

be assumed that the casein alone digests in the mixture since the rate of hydrolysis of the casein is the same in the mixture as in the pure casein while the increase in formol titration is much greater in the

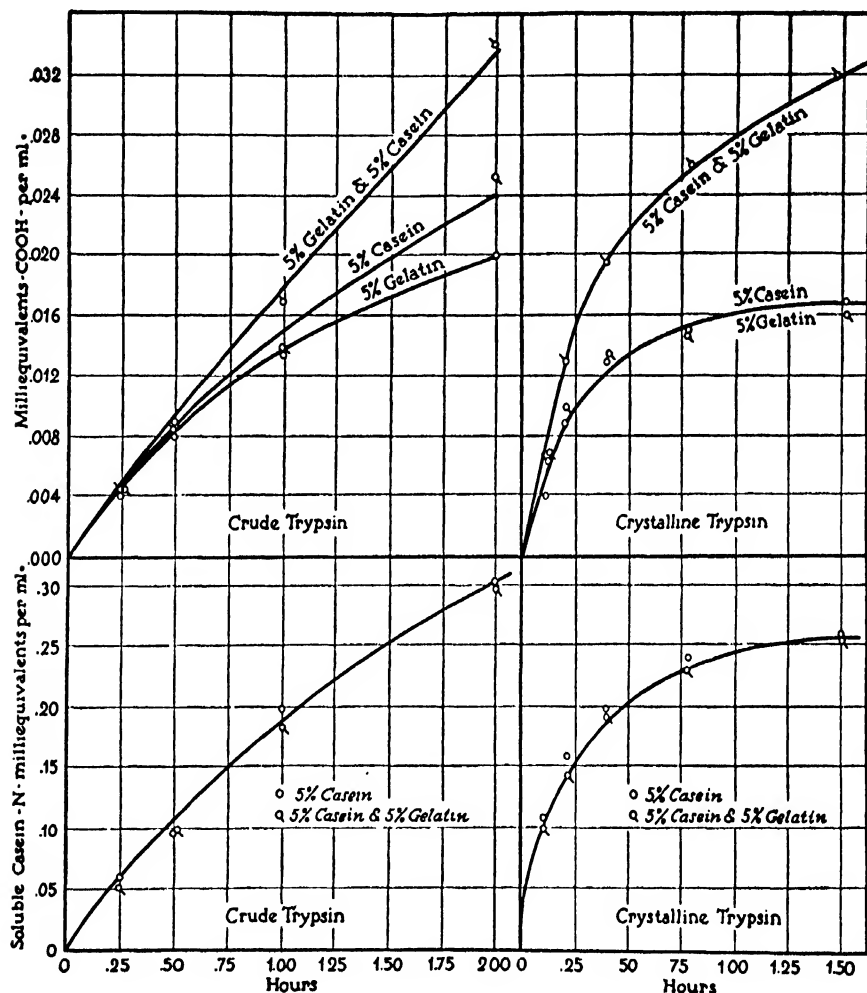


FIG. 3. Digestion of mixtures of casein and gelatin with crude and crystalline trypsin. Upper figures show increase in formol titration of mixture and of two proteins separately and lower figures show digestion of casein when present alone and in presence of gelatin.

mixture than in the casein solutions alone; also, the viscosity of the gelatin-casein mixture drops rapidly. These results show that the gelatin as well as the casein is hydrolyzed in the mixture.

*Kinetics of the Reaction as Determined by Changes in Viscosity*

The preceding experiments show that the abnormalities of the reaction between trypsin and proteins are less marked when purified trypsin solutions are used. The purified trypsin preparations contain a smaller number of enzymes than the crude preparations and the reaction does not proceed nearly so far with the purified as with the crude enzyme (9). These results indicate that the abnormal character of the reaction is due to the presence of a series of consecutive reactions rather than to the formation of an intermediate enzyme substrate compound and this conclusion is strengthened by the results of the experiments on mixtures of proteins. If this explanation is correct it would be expected that the results would agree better with those predicted from the simple theory of catalysis if the reaction were followed by a method which determined only the first step in the digestion and which was not affected by the subsequent reactions. The change in viscosity of the protein solutions offers such a method since the protein molecule itself is almost entirely responsible for the high viscosity and the viscosity is changed very slightly, if at all, by subsequent further hydrolysis. The method has the disadvantage that the physical significance of the change in viscosity is somewhat uncertain. The value for the viscosity itself cannot be used since this does not increase in proportion to the protein concentration and has no simple physical significance. All theories of viscosity agree that the viscosity is some function of the volume occupied by the solute, and Kunitz (14) has found an empirical equation which gives very reasonable values for the volume of the solute calculated from the viscosity. In calculating the results of the viscosity measurements, therefore, the values for the volume of the solute occupied by the hydrated protein molecules have been interpolated from the observed viscosity of Kunitz's equation. These values have then been corrected for the final volume occupied by the solute at the end of the reaction. The results of an experiment calculated in this way for the hydrolysis of casein with purified trypsin are shown in Table II. The values under  $K$  are those for the monomolecular equation using common logarithms and time in hours. They are reasonably constant and furthermore the value of the constant is about the same for 3 per cent and 5 per cent casein; *i.e.*, the reaction



TABLE II

*Kinetics of Digestion of Various Concentrations of Casein with Crystalline Trypsin as Determined by Changes in Viscosity*

$$K = \frac{1}{T \text{ hrs.}} \log_{10} \frac{V_o - V_e}{V_t - V_e}$$

Time <i>hrs.</i>	5 per cent casein				3 per cent casein			
	$\eta_s$	$V$	$V - V_e$	$K$	$\eta_s$	$V$	$V - V_e$	$K$
0	2.30	17.3	9.4		1.57	9.5	4.4	
0.16	1.975	14.3	6.4	1.00	1.463	8.1	3.0	1.0
0.22	1.905	13.6	5.7	1.00	1.424	7.7	2.6	1.0
0.63	1.686	11.1	3.2	0.80	1.34	6.4	1.3	0.85
1.71	1.532	9.2	1.3	0.75	1.288	5.5	0.4	0.62
25.00	1.439	7.9			1.282	5.4		

TABLE III

*Kinetics of the Hydrolysis of Various Concentrations of Gelatin with Crude and Purified Trypsin as Determined by Changes in Viscosity*

$$K = \frac{1}{T \text{ hrs.}} \log_{10} \frac{V_o - V_e}{V_t - V_e}$$

Concentration gelatin, per cent		5				3				1			
	Time	$\eta_s$	$V$	$V - V_e$	$K$	$\eta_s$	$V$	$V - V_e$	$K$	$\eta_s$	$V$	$V - V_e$	$K$
	<i>hrs.</i>												
Crystalline trypsin	0	4.13	27.5	20.7		2.31	17.4	12.9		1.285	5.4	3.6	
	0.20	3.10	22.5	14.7	0.70	1.94	14.0	9.5	0.65	1.23	4.5	2.7	0.63
	0.43	2.55	19.0	12.2	0.54	1.67	10.9	6.4	0.70	1.17	3.7	1.9	0.64
	0.67	2.25	16.8	10.0	0.48	1.55	9.3	4.8	0.64	1.15	3.1	1.3	0.66
	1.18	1.98	14.4	7.6	0.40	1.43	7.7	3.2	0.52	1.12	2.8	0.8	0.50
	2.66	1.68	11.1	4.3	0.26	1.33	6.0	1.5	0.69	1.115			
	48.00	1.37	6.8	0		1.23	4.5			1.092	1.8		
Crude trypsin	0	3.75	26.0	19.0		2.19	16.3	11.7		1.26	5.1	3.3	
	0.20	3.19	23.1	16.1	0.36	1.975	14.3	9.7	0.40	1.218	4.5	2.7	0.43
	0.95	2.50	18.8	11.8	0.22	1.69	11.2	6.6	0.25	1.16	3.5	1.7	0.30
	2.00	2.15	16.0	9.0	0.17	1.54	9.3	4.7	0.20	1.137	3.0	1.2	0.22
	4.00	1.91	13.6	6.6	0.11	1.44	7.8	3.2	0.15	1.115	2.5	0.7	0.16
	48.00	1.40	7.0	0		1.24	4.6			1.09	1.8		

is normal or nearly so when followed in this way. The results of an experiment with 1, 3, and 5 per cent gelatin digested with crystalline

and crude trypsin have been calculated in the same way (Table III). In this case, also, the value for the monomolecular constant is independent of the protein concentration as would be expected for a simple monomolecular reaction and in the case of crystalline trypsin the constant decreases only slightly during the first 50 per cent of the reaction. With crude trypsin the constant decreases more rapidly during the course of the reaction but still has about the same value for the different concentrations of protein. These experiments were carried out with about the same concentration of enzyme as were the preceding ones so that the difference is not due to differences in enzyme concentration.

### *Experimental Procedure*

*Protein Solutions.*—Isoelectric gelatin was prepared as described by Northrop and Kunitz (15). The casein used was Kahlbaum "casein according to Hammarsten." 25 gm. of the protein were dissolved in 400 ml. of  $M/10$  pH 7.6 phosphate buffer and the resulting solution titrated with alkali to pH 7.6 and made up to 500 ml. with  $M/10$  pH 7.6 phosphate buffer. Solutions of lower concentrations were made by dilution of this 5 per cent solution with  $M/10$  pH 7.6 phosphate buffer so that the solutions used were at pH 7.6 and contained  $M/10$  phosphate buffer.

*Preparation of Hemoglobin Solution.*—Crystalline hemoglobin solution dialyzed free from sulfate at the isoelectric point, titrated to pH 8.6, and diluted to 5 per cent hemoglobin concentration. Total alkali concentration about 0.02 normal. The flask containing the solution was immersed in boiling water for  $\frac{1}{2}$  hour to denature the hemoglobin since trypsin does not attack native hemoglobin. 2.5 per cent hemoglobin solution prepared from this solution by dilution with water.

The trypsin preparations were prepared from pancreatic pressed juice as described in a previous paper (9).

The formol titration was determined on 2 ml. of the protein solutions with  $N/50$  sodium hydroxide as described previously (16).

*Determination of Soluble Protein Nitrogen.*—1 ml. of the digestion mixture was added to 10 ml. of 2.5 per cent trichloroacetic acid; the suspension warmed to 70°C. for 10 minutes, cooled to 20°C. for  $\frac{1}{2}$  hour, and centrifuged. The precipitate was washed once with 10 ml. of 2.5 per cent trichloroacetic acid, centrifuged again and the precipitate dissolved in 2 ml. of  $N/10$  sodium hydroxide, and reprecipitated by the addition of 10 ml. 2.5 per cent trichloroacetic acid. The suspension was centrifuged, the precipitate dissolved in 1 to 2 ml. of  $N/10$  sodium hydroxide, and the solution made up to 10 ml. The nitrogen in 5 ml. of this solution was then determined by micro Kjeldahl. This gives the amount of insoluble casein nitrogen. The quantity of soluble nitrogen is then found by subtracting the figure for the soluble nitrogen from the total amount of nitrogen present at the beginning of the reaction. All reactions were carried out at 35°C.

## SUMMARY

The rate of digestion, as determined by the increase in non-protein nitrogen or formol titration, of casein, gelatin, and hemoglobin with crystalline trypsin preparations increases nearly in proportion to the concentration of protein, but with crude pancreatic extract the rate of digestion becomes independent of the protein concentration in concentrations of more than 2.5 per cent. With both enzymes the rate of digestion of mixtures of 5 per cent casein and gelatin is greater than would be expected from the point of view of a compound between enzyme and substrate. The rate of digestion of 5 per cent casein in the presence of 5 per cent gelatin is exactly the same as that of 5 per cent casein alone. This result is obtained with both enzymes. The digestion of casein with crude trypsin follows the course of a monomolecular reaction quite closely while with purified trypsin the velocity constant decreases as the reaction proceeds. In the case of hemoglobin the monomolecular velocity constant decreases with both purified and crude enzyme.

When the reaction is followed by changes in the viscosity of the solution the abnormal effect of changing substrate concentration disappears and the reaction is in fair agreement with the monomolecular equation. The results as a whole indicate that the abnormalities of the reaction are due to the occurrence of several consecutive reactions rather than to the formation of a substrate enzyme compound.

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# PHOTOKINESIS AND TONIC EFFECT OF LIGHT IN UNIONICOLA

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Orientation responses of plants and animals to light are often related in a simple direct manner to the light intensity or to the logarithm of the light intensity. If the reaction is directly proportional to the light intensity and to time the reaction is often said to obey the Bunsen-Roscoe law; if proportional to the logarithm of the light intensity the response has usually been considered to agree with the so called Weber-Fechner law. In the case of free-moving organisms, responsive to light, it has been debated whether light had any appreciable effect other than on primary orientation. Certain investigators, notably Patten (1917), Moore and Cole (1921), and Cole (1922), found a kinetic effect of light, while Dolley (1917) and Mast and Gover (1922) failed to find an effect of light intensity on rate of progression and denied the probability that there was such an effect.

It has been demonstrated in the case of larvae of the mussel-crab *Pinnotherecs maculatus* (Welsh, 1932) that light has a decided effect on rate of locomotion, and that over a certain range of intensities the velocity of progression increases as a definite function of the light intensity. The relationship, however, is not simple and obeys neither the Bunsen-Roscoe law nor the so called Weber-Fechner law.

Speed of progression in a free-moving organism is due to a combined effect of amplitude and frequency of movement of locomotor appendages, or of muscles concerned in locomotion. One or both of these factors might change with changing intensity of illumination, and the relation of either one to intensity might perhaps be simpler than that between velocity of progression and intensity. A change in amplitude of leg movement dependent upon light intensity would be due to a change in muscle tonus, thus lending support to the theory

upheld by Loeb (1918), Garrey (1918-19), Crozier and Cole (1923), Crozier and Federighi (1924-25, *a* and *b*), to cite only a few of the instances where differential muscle tonus, due to light, has been proposed or definitely shown to account for posture and orientation. The question of frequency and extent of muscular contraction, and of posture, as related to the external stimulus, is important for the understanding of tropistic behavior and it was considered desirable to study further the photokinetic effect of light.

The swimming appendages of *Pinnotheres* larvae are too small to be easily seen and another more suitable form was selected. The water mite, *Unionicola ypsilophorus* var. *haldemani* Piers, found living as a parasite on the gills of *Anodonta cataracta* Say is, when free from host material, distinctly positive to light (Welsh, 1930). It progresses by using only the middle two of four pairs of legs; the first pair is extended forward, the fourth pair drags behind as balancers. When these animals travel in water, on a suitable surface such as ground glass, their rate of locomotion is very constant in light of a constant intensity, and they proceed in a straight line toward a source of light. It is possible to obtain uniform data on velocity of progression as a function of light intensity and at the same time to determine the frequency and extent of leg movements.

The experiments were carried on under essentially the same conditions as in the work on the larvae of *Pinnotheres*. The mites were placed individually in a glass trough  $30 \times 4 \times 4$  cm., having a ground glass bottom and polished sides. The bottom was divided into 5 cm. lengths. The trough was partially submerged in a water bath kept at  $18.7^{\circ}\text{C.} \pm 0.2^{\circ}$  to eliminate temperature changes. The light sources were 6 volt, ribbon filament lamps, one at either end of the tank, arranged to yield parallel beams of light of the dimensions of the inside of the trough. The illumination toward which the animals were attracted and adapted before each trial was 21.7 foot candles at the center of the trough. The variable light source, without filters, gave an illumination of 255 foot candles at the middle of the trough, and by means of Wratten neutral tint filters this could be reduced to 68.0, 10.6, 1.95, or 0.185 foot candles.

In a test an animal was attracted toward the adapting light and after 2 to 3 minutes the light beams were reversed, the animal allowed to travel 10 cm., and then timed for the next 10 cm. With this procedure, time consumed in primary orientation did not enter, and a uniform rate of movement had been acquired before measurement was begun. Leg movements could be counted easily at the four higher intensities, but below 1.95 foot candles, while it was possible to see the animals clearly, it was not possible to count the leg movements.

Table I gives velocities, expressed as the reciprocal of the time to travel 1.0 cm., for three individuals. These have been selected as representative of several series which it was found inadvisable to average. Each velocity is the average of five determinations at a

TABLE I

Velocities of progression (centimeters per second) for three individuals at five different intensities of light. Temperature  $18.7^{\circ} \pm 0.2^{\circ}$ .

Illumination (foot candles)	Series 1	Series 2	Series 3
0.185	0.318	0.333	0.369
1.95	0.338	0.351	0.376
10.6	0.352	0.375	0.391
68.0	0.392	0.407	0.400
255.0	0.410	0.420	0.408

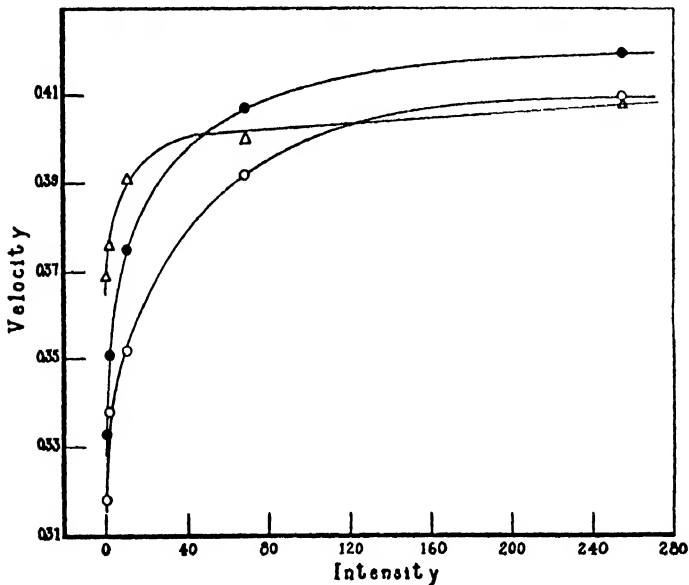


FIG. 1. Data of Table I plotted as velocity (cm. per second) against illumination (foot candles). Series 1, represented by open circles, and Series 2, represented by closed circles, were begun at the highest intensity. Series 3, represented by triangles, was begun at the lowest intensity.

given intensity. Series 1 and 2 were begun at the highest intensity and the illumination was diminished by successive steps; Series 3 was begun at the lowest intensity and the intensity was increased. As may be seen in Fig. 1, the results for the three mites are not identical,

although in each case when velocity is plotted against intensity a smooth curve is obtained; the curves are essentially similar in shape but are far from straight lines. When the same data are plotted as velocity against the logarithm of the light intensity (Fig. 2) sigmoid curves are obtained, and it is more evident that there are minimum and maximum velocities below and above which changes in light intensity have no effect. It is quite possible that in instances where

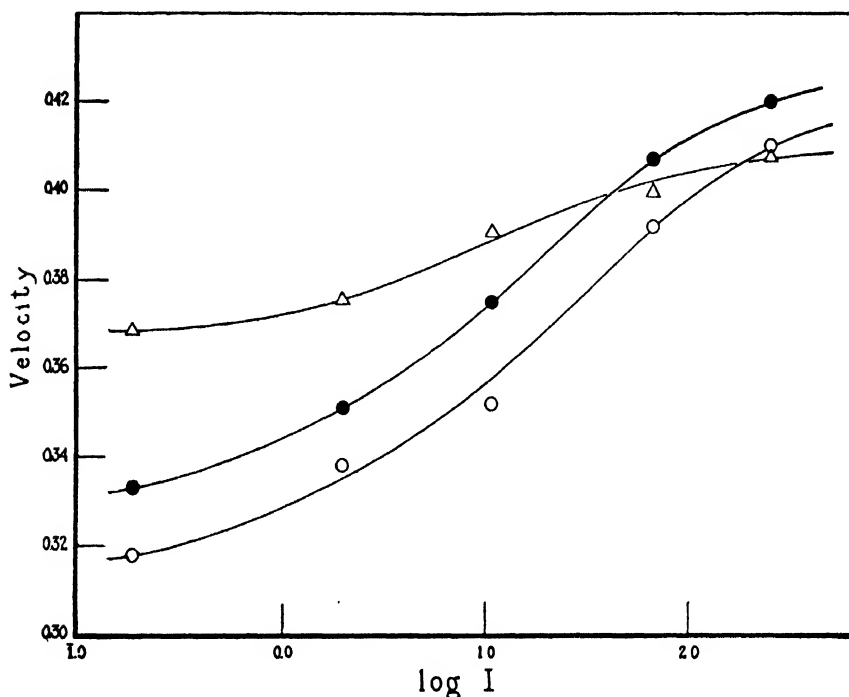


FIG. 2. Same data as in Fig. 1 plotted as velocity of movement against the logarithm of the light intensity.

such a relationship has been found to be rectilinear, and therefore considered to obey the Weber-Fechner law, that a middle range of intensities has been used which would yield essentially a straight line when rate of progression was plotted against the logarithm of light intensity. It is also possible that in cases where light has apparently had no effect on rate of progression a range of intensities above that necessary to elicit a maximum response has been used. It is evident that there is here no very simple relationship between speed of progression and light intensity; but as the "speed" is the result of a combined effect of

light intensity; but as the "speed" is the result of a combined effect of amplitude of stride and frequency of stepping one would not necessarily expect a simple relation.

If we examine the data on number of leg movements as a function of light intensity (Table II) it is interesting to note that as the intensity increases and the velocity likewise increases, the number of leg movements per 10 cm. path actually *decreases*. This indicates that the length of stride must increase with increasing intensity of illumination, while the frequency might or might not change. As may be seen in Table II, both amplitude of stride and frequency of leg movements increase as certain functions of light intensity. Fig. 3 shows plots of averages of both amplitude and frequency against the logarithm of the light intensity. Amplitude of stride is seen to be directly proportional

TABLE II

Numbers of leg movements in travelling 10 cm.; average length of stride; and frequency of leg movement, at four intensities of illumination.

Illumination (foot candles)	No. of leg movements per 10 cm. path				Length of stride	Frequency
	Series 1	Series 2	Series 3	Average		
					cm.	
1.95	60.1	60.2	59.6	59.96	0.167	2.125
10.6	58.4	59.2	57.2	58.26	0.172	2.162
68.0	56.2	57.4	56.0	56.53	0.177	2.260
255.0	55.2	56.0	54.5	55.23	0.181	2.281

to the logarithm of the light intensity, as the plot yields a straight line. Frequency of stepping, however, does not bear such a simple relation and yields a sigmoid curve. Combining the effects of two factors yields the velocity curves of Fig. 2, and accounts for the shape of the curves in Fig. 2.

A somewhat similar case was investigated by Crozier and Stier (1925-26). These authors found that the relation between temperature and speed of progression in *Malacosoma* larvae was not a simple one, but that when frequency and amplitude of movement of the anal prolegs were determined as functions of temperature the frequency was found to vary directly with the temperature while the amplitude of steps did not. It is possible that such a relation exists between leg movements and temperature in *Unionicola*.



The change in length of stride in *Unionicola* is due to tonus changes in the leg muscles; this is due to the tonic effect of light, and is directly proportional to the logarithm of the light intensity over the range employed. Such effects have been studied previously chiefly during orientation, or during the exhibition of circus movements resulting from blinding one eye. In any case, the muscle tonus theory upheld by Loeb and others seems the only explanation of the observed effects.

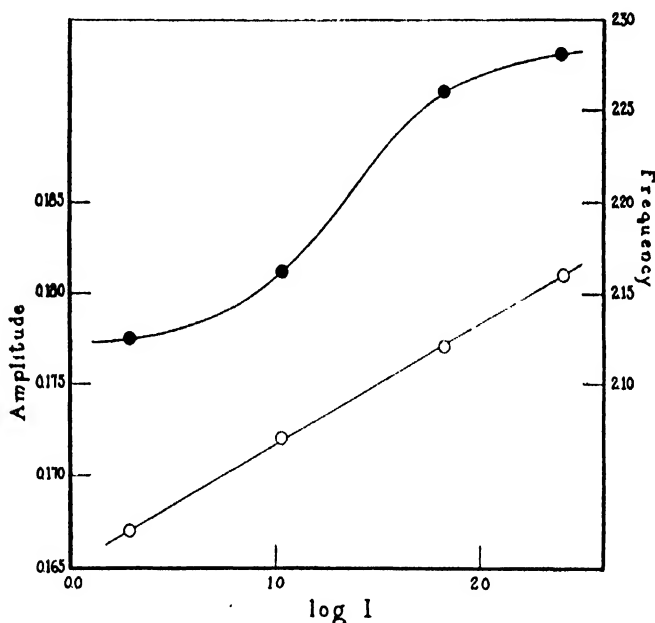


FIG. 3. Averages of amplitude of stride (open circles), and frequency of leg movement (closed circles), as function of the light intensity. Data from Table II. Combining these curves would produce a curve representing the average of those shown in Fig. 2.

#### SUMMARY

1. The speed of progression of *Unionicola*, a water mite, is influenced by light; and over a certain range increases as a function of the light intensity.

2. The relation between speed and light intensity is not a simple one, as the speed of progression is due to the combined effect of amplitude of steps and frequency of leg movement.

3. The amplitude of stride increases in direct proportion to the logarithm of the light intensity, while the frequency of stepping has no such simple relation to intensity.

4. The change in length of stride with changing light intensity indicates a tonic effect of light on the locomotor muscles. Such an effect has been observed previously in studies of orientation, due to unequal illumination, which produces changes in posture.

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# THE COMBINATION OF CERTAIN PROTEINS WITH HYDROCHLORIC ACID

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During the past few years the writer has obtained electromotive force measurements of cells without liquid junction of the type

(+) Ag, AgCl, HCl (0.1 M) + protein, H<sub>2</sub> (-)

with the object of determining the maximal combining capacities of various proteins for hydrochloric acid. The results of such measurements with edestin (1) and gelatin (2) were interpreted as indicating that the protein exhibited a definite maximal combining capacity for each of the ions of the acid, combining with considerably more hydrogen ion than chloride ion. In arriving at this conclusion it was assumed that the mean activity coefficient of the hydrogen and chloride ions was not affected by the presence of the protein, except for the small decrease in ionic strength due to the combination of part of the protein with both ions. Similar measurements with casein have not hitherto been submitted for publication because no way could be found to interpret them on the basis of these assumptions. It is now possible to interpret these data, as well as the experiments with edestin and gelatin, by assuming that none of the proteins combines at all with chloride ion, that each protein has a definite maximal combining capacity for hydrogen ion, and that the mean activity coefficient of the ions of hydrochloric acid is decreased by the presence of the protein in such a way that its logarithm is a linear function of the protein concentration.

The latter assumption was suggested by the recent results of Failey (3), who measured the solubility of thallos chloride in solutions of nitric acid containing varied amounts of edestin. Without assuming any combination of ions with the protein, he calculated mean activity

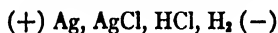
coefficients of thalious chloride from his solubility data, and found that these coefficients varied in the way just described, which may be represented by the equation

$$-\log \gamma = -\log \gamma_0 + Bg. \quad (1)$$

In this equation  $\gamma$  is the mean activity coefficient of a strong electrolyte in a protein solution,  $\gamma_0$  its coefficient in a corresponding solution without protein,  $g$  is the protein concentration in gm. per 1000 gm. of water, and  $B$  is a positive number, a constant for each protein. Failey also pointed out that the writer's data on gelatin and edestin could be interpreted, without the assumption of combination of the protein with chloride ion, by applying this linear relation to variations in the activity coefficient of the chloride ion only. Since there is no way of measuring with certainty the activity coefficient of a single type of ion, it seemed desirable to find out whether the data could be interpreted by applying Failey's rule to the mean activity coefficient of the hydrogen and chloride ions.

#### EXPERIMENTAL

The experiments with edestin (1) and gelatin (2) (Eastman Standard) have already been described in detail. Table I of the edestin paper (1) contains data obtained with hydrochloric acid free from protein, which were extrapolated by the method described elsewhere (4), giving the value  $E_0 = 0.2193$  volt for the cell



at 30°C. These data were also used to obtain, by a short extrapolation, the values of the activity coefficient  $\gamma_0$  of the protein-free acid. The negative logarithms of these values are given in the fifth column of Table I.

Two preparations of casein were used. Casein I had been prepared from milk<sup>1</sup> by the method of Northrop (5), which involves the filtration of an acid solution and the rejection of the insoluble portion. Such preparations probably correspond to some of the more acid-soluble fractions obtained by Linderstrøm-Lang (6). Its ash content was about 0.5 per cent of the dry protein. Solutions were prepared and concentrations calculated by the method used with edestin (1). The electrodes were prepared by the method previously described (7) and the E.M.F. was measured at 30°C. as before.<sup>2</sup>

<sup>1</sup> This casein was prepared by Mr. C. E. Heinrichs, formerly technician in the Laboratories of The Rockefeller Institute.

<sup>2</sup> These experiments were carried out by Miss Esther R. Mason, formerly research assistant in this Laboratory.

Casein II was prepared, as in the original method of Hammarsten (8), by precipitation from skimmed milk with dilute acetic acid. The casein was washed by decantation and dissolved by adding only enough dilute sodium hydroxide (0.02 *M*) to keep the pH below 7.0, as suggested by Cohn (9). The solution was filtered through paper pulp and treated with a dilute hydrochloric acid solution (0.05 *M*) equivalent to twice the amount of alkali present. This solution was added rapidly with violent stirring, in order to carry the reaction past the isoelectric point without causing the casein to precipitate. This acid solution (pH about 2.6), although quite clear, was again filtered through pulp, and the final precipitation was made by slowly adding dilute sodium hydroxide, as suggested by Northrop (5). The precipitated casein was washed by stirring and decantation until no appreciable change was found in the conductivity of successive wash waters, after which the casein was filtered off and dried with alcohol and ether. This preparation was much more difficult to dissolve in hydrochloric acid than was casein I. It was possible to obtain solutions only as concentrated as 5 per cent by treating the dried casein first with 0.01 *M* hydrochloric acid, making use of the finding of Loeb and Loeb (10) that casein dissolves most rapidly in acid of about this concentration. The acid concentration was then brought up to 0.1 *M* by adding known amounts of a 0.2 *M* solution and of water. The casein concentrations were known from dry weight determinations at 105° on the powdered preparation. In making the E.M.F. measurements, freshly plated electrodes were used for each solution.<sup>3</sup>

#### RESULTS AND DISCUSSION

The results obtained with the four protein preparations are given in Table I. The electromotive force of such cells is given by the equation

$$E = E_0 - 0.06015 \log m_H m_{Cl} \gamma^2. \quad (2)$$

Since there is no independent evidence for the combination of chloride ion from hydrochloric acid with a protein in solution,  $m_{Cl}$  is taken as identical with  $m$ , the total molality of the acid. It is generally believed that a protein in acid solution combines with hydrogen ion. This belief rests on reasoning by analogy with the behavior of amino acids, and on the results of indicator experiments, pH measurements with the hydrogen electrode, conductivity measurements, and cataphoresis measurements. If this assumption is correct, the molality of free hydrogen ion,  $m_H$ , is equal to  $m - gx$ , where  $m$  is the total molality

<sup>3</sup> This work was done by Mr. B. W. Eno and Mr. A. E. Benaglia, formerly technicians in this Laboratory.

TABLE I

*Electromotive Force at 30°C. of the Cells Ag, AgCl, HCl + Protein, H<sub>2</sub>*

Protein	<i>m</i>	<i>g</i>	<i>E</i>	$-\log \gamma_0$	$-\log \gamma$	$10^4 B$	$10^4 x$
Edestin. Reference 1	0.1007	13.05	0.3568	0.096	0.105	8.0	13.4
	0.1020	18.55	0.3592	0.096	0.111		
	0.1050	42.35	0.3732	0.097	0.132		
	0.1055	62.70	0.3961	0.097	0.147		
Gelatin. Reference 2	0.1000	50.3	0.3708	0.096	0.116	4.0	9.6
	0.1000	70.4	0.3841	0.096	0.125		
	0.1000	90.5	0.4084	0.096	0.131		
Casein I	0.1028	11.2	0.3534	0.097	0.107	9.5	8.0
	0.1054	29.2	0.3585	0.097	0.126		
	0.1060	46.6	0.3652	0.097	0.144		
	0.1093	66.3	0.3715	0.098	0.160		
	0.1105	82.4	0.3795	0.099	0.178		
	0.1077	83.4	0.3821	0.098	0.176		
Casein II	0.1000	20.0	0.3579	0.096	0.114	8.8	8.0
	0.0999	30.0	0.3615	0.096	0.122		
	0.0999	40.0	0.3654	0.096	0.131		
	0.1000	50.0	0.3698	0.096	0.140		

*m* = molality of HCl.*g* = gm. protein per 1000 gm. water.*E* = observed E.M.F. in volts, corrected to 1 atm. dry H<sub>2</sub>. $\gamma_0$  = activity coefficient of pure HCl of molality *m*. $\gamma$  = activity coefficient of HCl in protein solution, calculated by the use of the selected value of *x*.*B* = slope of line obtained by plotting  $-\Delta \log \gamma$  against *g*, using the selected value of *x*.*x* = maximum number of equivalents of H<sup>+</sup> combined with 1 gm. protein, selected to give the best straight line in the above plot.

of the acid, *g* is the protein concentration in gm. per 1000 gm. of water, and *x* is the number of equivalents of hydrogen ion bound by 1 gm. of protein. Equation (2) then becomes

$$E = E_0 - 0.06015 \log m (m - gx) \gamma^2. \quad (3)$$

By assuming values for *x* it is possible to calculate corresponding values for  $\gamma$  from each measurement of E.M.F., since *E*<sub>0</sub>, *m*, and *g* are known.

If Failey's rule applies, a straight line should be obtained on plotting  $-\log \gamma$  against  $g$  only when  $\gamma$  has been calculated by the use of the correct value for  $x$ . The data have been plotted in this way in Figs. 1 to 4. Since the acid concentrations differed somewhat from 0.1 M, the

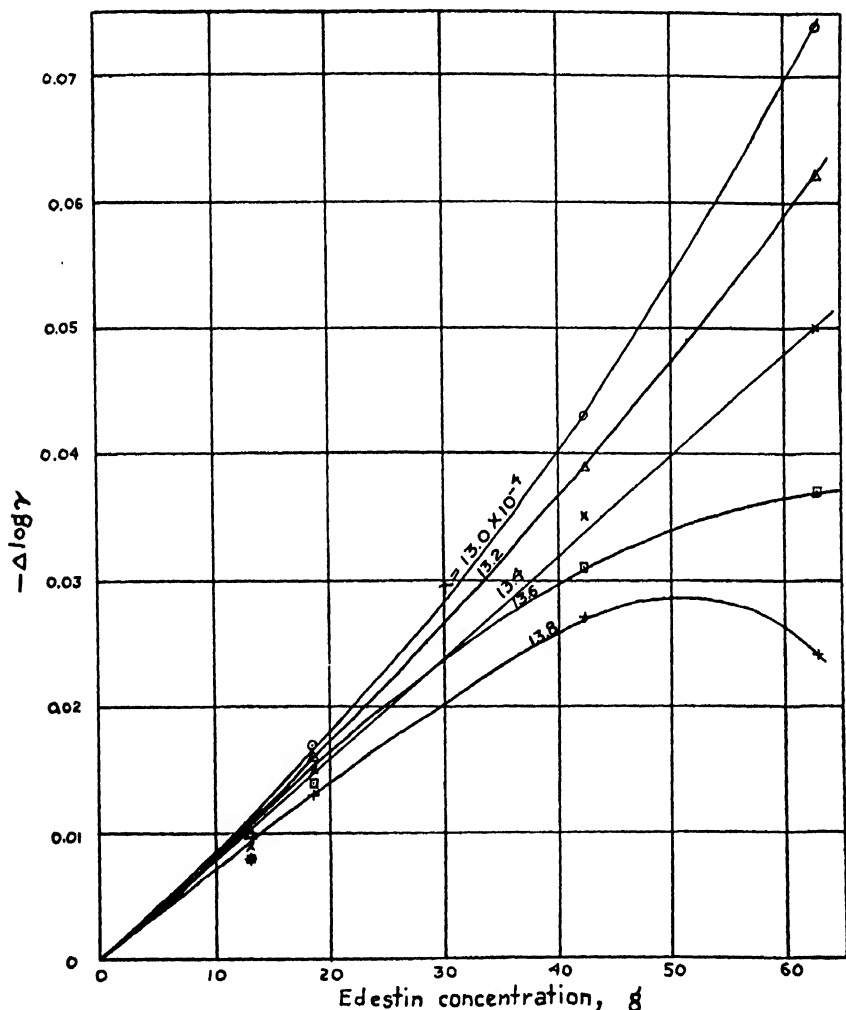


FIG. 1. Edestin + HCl. Plot of  $-\Delta \log \gamma$  against  $g$ . A straight line is obtained only if  $x = 13.4 \times 10^{-4}$ .

ordinates of the figures are not  $-\log \gamma$ , but  $-\Delta \log \gamma$ , which is  $-\log \gamma + \log \gamma_0$ . According to Failey's rule this difference should be directly proportional to the protein concentration, and whether this rule holds or not the curves must all pass through the origin.



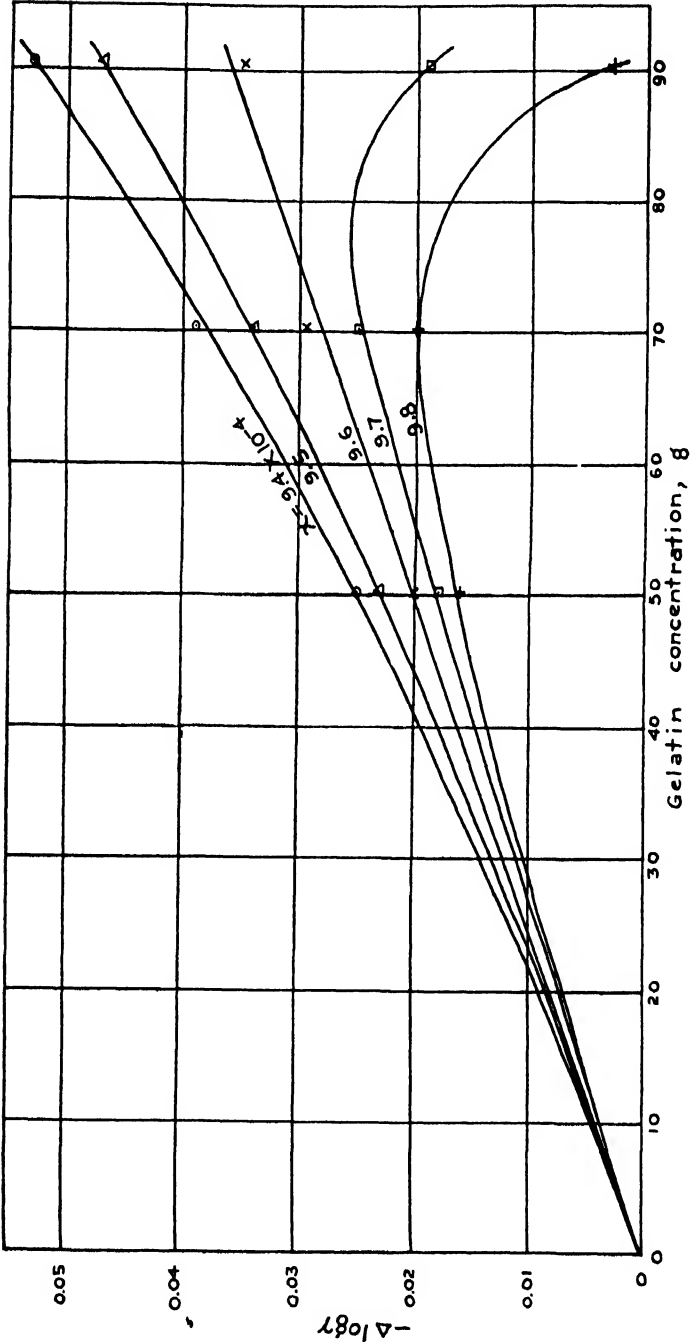


Fig. 2. Gelatin + HCl. Plot of  $-\Delta \log \gamma$  against  $g$ . A straight line is obtained only if  $x = 9.6 \times 10^{-4}$

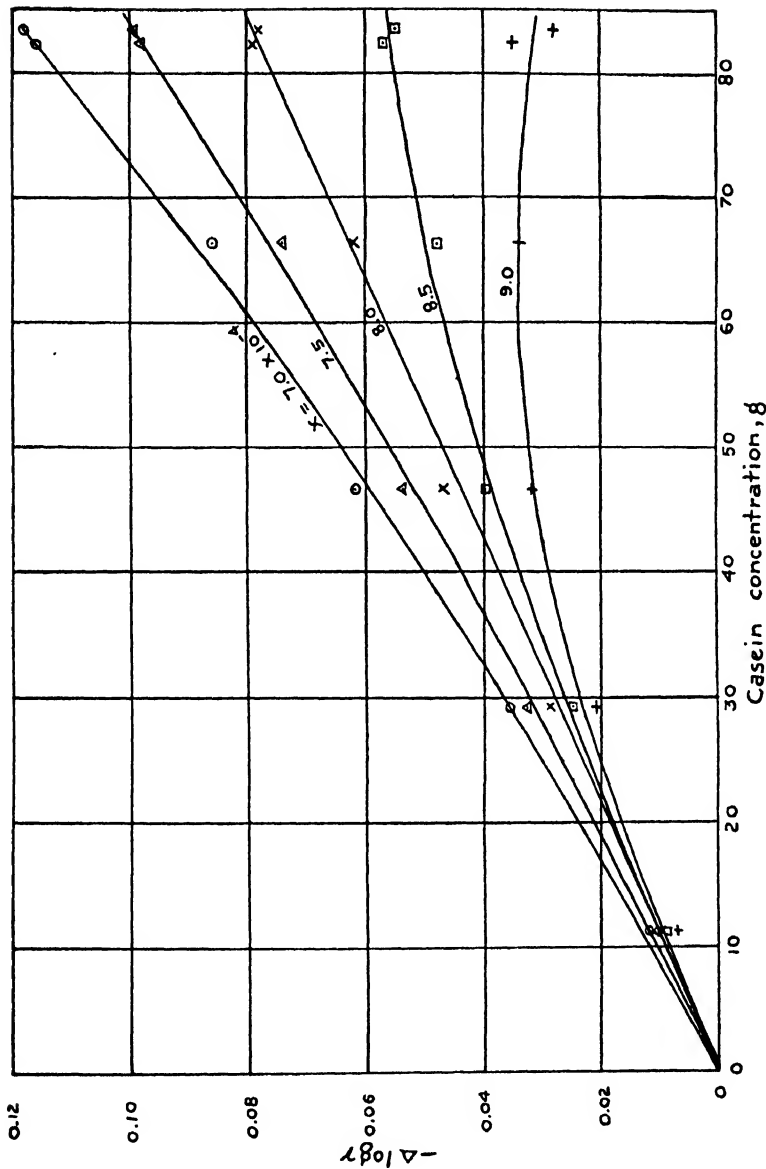


FIG. 3. Casein I + HCl. Plot of  $-\Delta \log \gamma$  against g. A straight line is obtained only if  $x = 8.0 \times 10^{-4}$ .

Fig. 1 shows that with edestin a straight line is obtained only if  $x = 13.4 \times 10^{-4}$ , with a possible uncertainty of  $\pm 0.1 \times 10^{-4}$ . Fig. 2 shows that the same is true for gelatin only if  $x = 9.6 (\pm 0.05) \times 10^{-4}$ . It is noteworthy that these figures for the numbers of equivalents of hydrogen ion combined with 1 gm. of protein are identical with those

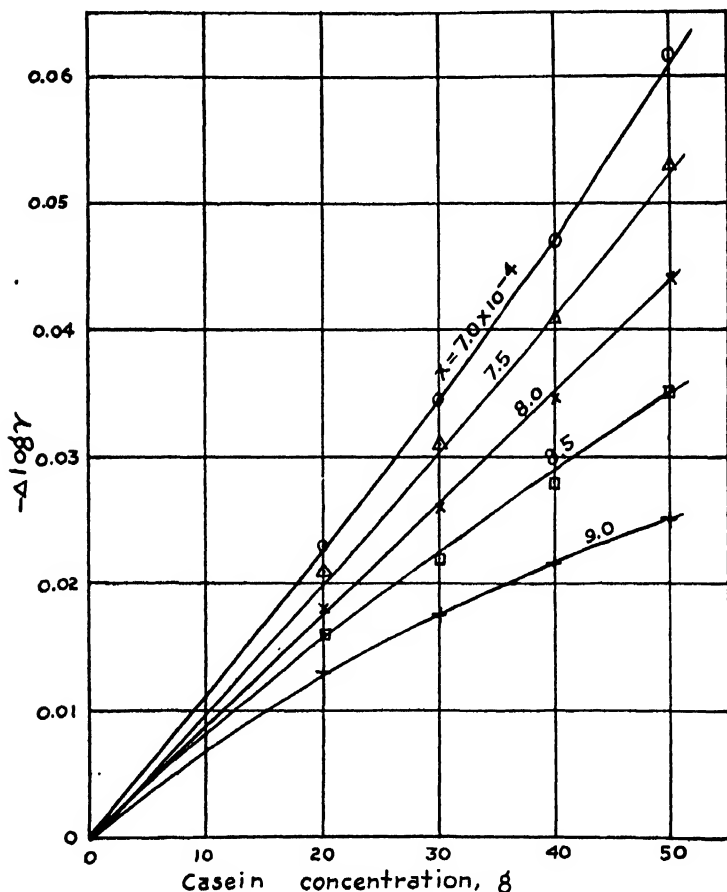


FIG. 4. Casein II + HCl. Plot of  $-\Delta \log \gamma$  against  $g$ . A straight line is obtained only if  $x = 8.0 \times 10^{-4}$ .

previously calculated from the same data (1, 2) on the basis of other assumptions.

Figs. 3 and 4 show that the data for casein are somewhat less definite, but in each case the points fall most nearly on a straight line if  $x = 8.0 (\pm 0.25) \times 10^{-4}$ . This value for the maximum combining

capacity of casein for hydrogen ion is intermediate between the values ( $6$  and  $9 \times 10^{-4}$ ) calculated by Cohn (11) in 1925 from compiled data on casein prepared in various ways. In the present experiments the method of preparation of the casein seems to have had no effect on the value of the combining capacity,  $x$ , but only on the value of  $B$ , the slope of the graph of equation (1).

The values of  $B$  which are given in Table I are those obtained by the use of the values of  $x$  selected as giving the best straight lines. These values are all considerably less than the slopes calculated by Failey (3), either from his own data or from the results of other workers. No reason is known for this discrepancy, except that Failey's calculations from the present writer's data are probably illogical because they involve contradictory assumptions as to the activity coefficients of the hydrogen and chloride ions.

By combining equations (1) and (3), and using the selected values of  $x$  and  $B$  and the experimental values of  $E_0$ ,  $\gamma_0$ ,  $m$ , and  $g$ , it is possible to obtain calculated values of  $E$ . The values obtained in this way are in fully as good agreement with the observed values as those previously published, but since the graphical method of Figs. 1 to 4 proves the same point somewhat more directly, it seems unnecessary to include the calculated values of  $E$  in Table I.

#### SUMMARY

Electromotive force measurements of cells without liquid junction, of the type  $\text{Ag}, \text{AgCl}, \text{HCl} + \text{protein}, \text{H}_2$ , have been made at  $30^\circ\text{C}$ . with the proteins gelatin, edestin, and casein in  $0.1 \text{ M}$  hydrochloric acid. The data are consistent with the assumptions of a constant combining capacity of each protein for hydrogen ion, no combination with chloride ion, and Failey's principle of a linear variation of the logarithm of the mean activity coefficient of the acid with increasing protein concentration. The combining capacities for hydrogen ion so obtained are  $13.4 \times 10^{-4}$  for edestin,  $9.6 \times 10^{-4}$  for gelatin, and  $8.0 \times 10^{-4}$  for casein, in equivalents of combined  $\text{H}^+$  per gm. of protein.

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# ON TEMPERATURE CHARACTERISTICS FOR DIFFERENT PROCESSES IN THE SAME ORGANISM

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## I

It is of interest for the theory of temperature characteristics of vital processes to test the assumption that in many cases the quantity  $\mu$  in the expression  $\ln K \propto \mu/RT$  may have a specific, local significance. One view in this connection has been expressed by Murray (1925-26) in a paper on the relation of temperature to the rate of contraction of embryonic heart muscle fragments of the chick in culture. He reported that he found no constancy in the values of the "temperature characteristics" ( $\mu$ , in the Arrhenius equation; cf. Crozier, 1924) and no constancy in the "latitude of variation" (Crozier and Federighi, 1924-25; Crozier and Stier, 1926-27, *a*) over any portion of the temperature range.

As an explanation for the reported lack of constancy in the values of the temperature characteristics Murray proposed that "possibly the regularity of the temperature effect as empirically determined upon certain functions *in vivo* depends to a greater extent than is acknowledged upon the integrity of the organization and the interrelationships of the parts of the whole, and less upon one particular chemical reaction or catalyst" (Murray, 1925-26, p. 788). The inadequacy of this notion has been discussed by Crozier and Stier (1926-27, *c*).

The suggestion might be understood in either of two ways. The "organization"—factor might pertain simply to the heart or other structure immediately implicated in the observations; or it might be taken as a feature of the organism as a whole. The former effect must be granted at once as an obvious truism, in the sense that the structure of a heart, for example, permits control by a definite localized pace maker. The latter view can be tested by determining simul-

taneously the temperature characteristics for two or more different activities in the same individual. Such experiments were made with *Asellus* (Crozier and Stier, 1926-27, *b*), determining synchronously the frequencies of heart beat and of respiratory movements in the same individual. It was found that the frequencies in these organs do not vary together, neither do they have the same temperature characteristic (*cf.* Fig. 7, Crozier and Stier, 1926-27, *b*). This holds also for the embryos of *Limulus*, the frequencies of gill movements yielding increments quite different from those obtained for the hearts (Crozier and Stier, unpublished).

The following experiments were made with more careful refinement of technique in order to test further the notion of whether the organism as a whole would determine one temperature characteristic for all its activities.

## II

The cladoceran *Daphnia* was chosen because its respiratory movements and its heart beat are easily visible through the transparent carapace; and since Cladocera reproduce parthenogenetically, one obtains successive broods of individuals unusually uniform genetically and highly comparable in development. The *Daphnia* used for the greater part of these experiments (*Daphnia magna*) were at least second generation descendants produced parthenogenetically from one individual. The stocks for these experiments were furnished by Dr. L. A. Brown from his highly "purified" cultures. We wish to thank him for his kindness to us during these experiments.

A single animal was placed in a small glass trough through which a small stream of oxygenated culture fluid (*cf.* Banta, 1921, for ingredients) was allowed to flow. Each individual was placed in a glass tube of a bore sufficient to hold the animal without pressure; then by rotating each tube the heart and "gills" were both visible when the illumination was properly adjusted from below. Culture medium through which air had been bubbled for 10 hours was run into each tube in a small stream through a small-bore glass nozzle. Light was admitted through a window in the bottom of the water thermostat into which the glass container holding the preparations could be lowered. The observing microscope and an objective used as a water immersion lens passed through the cover of the vessel holding the animals, also a thermometer reading to  $0.01^{\circ}$ . The temperature of the water bath was held constant to within  $\pm 0.01^{\circ}\text{C}$ . A thermostat similar to the one described by Crozier and Stier (1926-27, *c*) was used for these experiments.

Time for ten to twenty movements of the heart and "gills" was taken simultaneously with stop-watches by two observers<sup>1</sup> who could view the same preparation by aid of a divided ocular ("demonstration ocular").

### III

The results of the experiments presented in Table I and Figs. 1 and 2 do not uphold Murray's contention of a control of the magnitude of temperature characteristics by the whole organism; the same value of  $\mu$  was not found for each of two processes tested simultaneously in the same organism. These differences of magnitudes of thermal increments for heart beat and respiratory movements respectively in *Daphnia* indicate that the frequencies of movements are controlled by different "governing reactions" (Crozier, 1924; 1925-26, *a*).

Not only do differences of temperature characteristic for these two processes exclude the possibility of the organism exerting some general kind of control, but there are differences in the behavior of the two organs pointing to an independence of direct control of one organ over the other. (1) Whenever "spontaneous movements" occur one can be sure to find some alteration in the frequency of gill movements, even as much as a temporary cessation; however, the heart beat usually continues with unchanged rhythm. (2) There are greater numbers of instances showing a "break" in the line relating frequency of respiratory movements to temperature than in the plots for heart beat. There are seven such instances for "gills" and only one for heart beat where  $\mu = 12,500$  above  $11.1^{\circ}\text{C}$ . and  $25,000$  below. (3) The absolute frequencies of heart beat and "gill" movements differ by about 100 per cent.

Average critical temperatures (Crozier, 1925-26, *b*) for respiratory activity occurred at  $3.4^{\circ}$ ,  $5.0^{\circ}$ ,  $11.2^{\circ}$ ,  $13.0^{\circ}$ ,  $14.4^{\circ}$ , and  $18.9^{\circ}$ . For some reason as yet unknown, these values do not agree closely (except at  $5.0^{\circ}$ ,  $14.4^{\circ}$ , and  $18.9^{\circ}$ ) with the modal values usually found for many biological processes (Crozier, 1925-26, *b*). Illustrations of other cases in which "atypical" values appear can be found in respiratory movements of decapitated grasshoppers (Crozier and Stier, 1924-25), and in the pulsations of "accessory hearts" in the amputated legs

<sup>1</sup> For assistance in these experiments we are indebted to Mr. T. Thornton Oxnard.



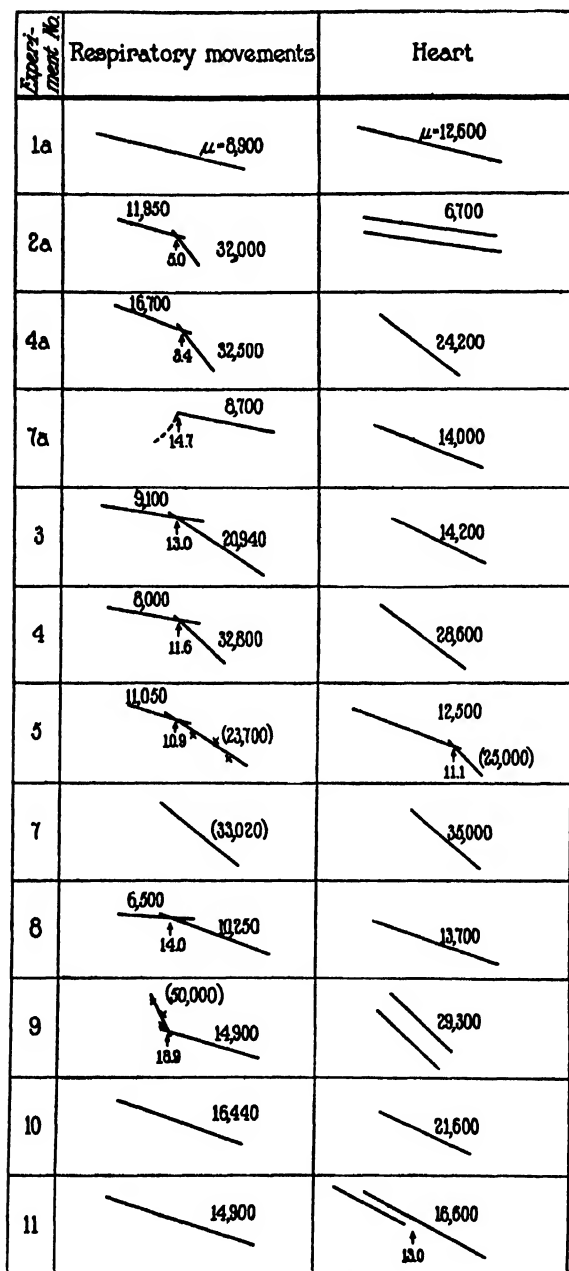


FIG. 1. All the graphs obtained from twelve experiments with *Daphnia* are given diagrammatically. The lines are based on six or more observations (each the average of at least ten stop-watch readings) spaced less than a degree apart. In cases where lines are based on fewer observations small crosses indicate the actual position of the observations in relation to the line.

of *Notonecta* (Crozier and Stier, 1926-27, b). In each of those cases progressive changes in the preparation are leading to death, so that

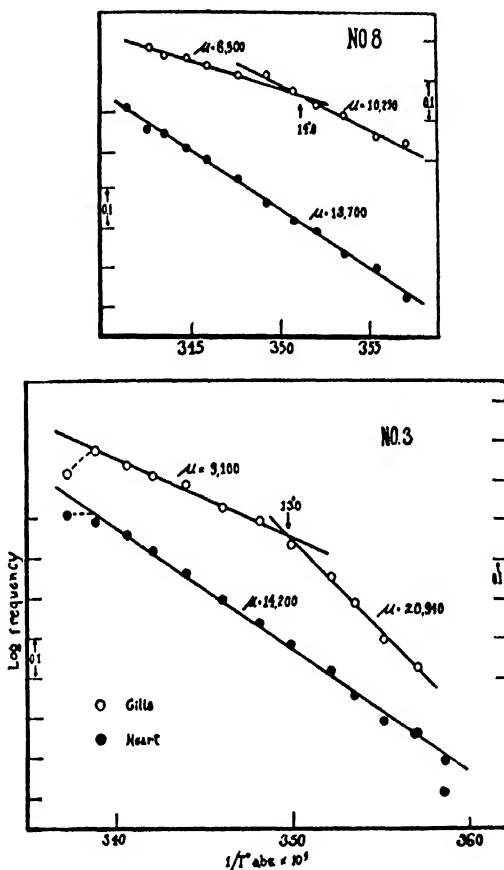


FIG. 2. Typical observations on frequency of heart beat and of respiratory movements made simultaneously by two observers at constant temperature, from two experiments with *Daphnia* over a wide range of temperature (cf. Fig. 1 for all other experiments). Open circles refer to "gill" movements, solid circles to heart beat. In each case there is a difference in the magnitudes of the temperature characteristics for heart beat and for gill movements. Another difference is found in the unbroken line relating frequency of heart beat to temperature, whereas for respiratory movements a "break" always occurs yielding two increments, one above the break and one below (cf. also Fig. 1). Two additional "breaks" occur above  $21.9^{\circ}\text{C}$ . in the graph for Experiment 3. Above this temperature thermal "destruction" occurs and consequently the observations for both processes fall below the lines fitted to the data in the lower temperature range. For additional illustrations of this type of "break," consult Crozier, 1925-26, a.

abrupt change of  $\mu$  comes about as a function of time, independently of change of temperature.

In *Daphnia* the critical thermal increment, 12,000 calories, appears only twice, and in each case it is obtained for frequency of heart pulsations, never for respiratory movements. This value has been frequently obtained for heart beat—rarely for respiratory movements (*cf.* Crozier, 1925-26, *b*).

TABLE I

Significant temperature characteristics based on six or more observations spaced less than a degree apart yield average values of  $\mu$ :

For respiratory movements	6,500.....	1 instance
	8,680.....	4
	11,080.....	3
	14,900.....	2
	16,550.....	2
	20,940.....	1
	32,430.....	3
For heart beat	6,700.....	1
	12,550.....	2
	13,970.....	3
	16,600.....	1
	21,600.....	1
	24,200.....	1
	28,950.....	2
	35,000.....	1

Sample experiments can be found in Fig. 2.

## IV

We did not obtain the same temperature characteristic for each of the two processes measured simultaneously in the same organism. Furthermore, our observations on the behavior of the two processes with reference to "spontaneous" movements, absolute magnitudes of their frequencies, critical temperatures, and the distinct differences in the kinds of curves obtained, lead to the conclusion that the organism as a whole has no control over the determination of temperature characteristics for its separate activities. It is assumed that the initiation of pulsation arises in each organ in a few groups of cells,—pace makers; the inner metabolic states of these cell groups are thought to

be differently adjusted so that each organ might yield independently any one of the modal thermal increments represented in the series of linked chemical reactions governing the frequency of contraction (*cf.* Crozier, 1924–25).<sup>2</sup>

It is highly probable that there may be instances when the same temperature characteristic would be obtained simultaneously for several processes in the same individual organism. Distribution polygons of critical increments obtained from data on heart beat and rates of many other vital processes were found by Crozier (1925–26, *a*) to be superimposable—the same modal values of the temperature characteristic being obtained for heart beat and for all other vital processes.

What determines the change from one temperature characteristic to another for any physiological activity within the organism? Can the organism by virtue of its own organization control the change from one governing reaction to another in all of its different processes? The data we have reported uphold the view that the critical increment for each process is dependent upon the metabolic state of the elements governing its activity (*e.g.*, the pace makers controlling the frequency of the heart beat); the temperature characteristic of a process is apparently independent of other processes in the same organism and is certainly not dependent upon a general “organization” factor.

#### SUMMARY

The temperature characteristics ( $\mu$ ) for two activities (heart beat and respiratory movements) studied simultaneously in the same individual organism (*Daphnia magna*) were always found to differ in magnitude. The type of graph obtained when the frequency of these movements was plotted according to the Arrhenius equation was also distinctly different for each activity. The organism therefore does not determine a uniform magnitude of the temperature characteris-

<sup>2</sup> In a recent investigation (Wolf, 1932–33) of the relation of temperature to the pulsation frequency of the advisceral and abvisceral heart beats of *Ciona intestinalis* the same temperature characteristics and critical temperature were obtained for advisceral and abvisceral beats. The pulsation frequency of ad- and abvisceral heart beat was the same at any temperature in any one individual. These findings indicate that the general metabolic condition of the two ends of the heart (pace makers) is the same in any one individual.

tic for each of its activities; the values of  $\mu$  must therefore have, to this extent, a local, specific meaning.

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## BIOELECTRIC POTENTIALS IN VALONIA

### THE EFFECT OF SUBSTITUTING KCl FOR NaCl IN ARTIFICIAL SEA WATER

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In a study of bioelectric potentials in *Valonia macrophysa*, the effects produced by KCl are especially interesting in view of the remarkable degree to which this salt is accumulated in the cell sap.<sup>1</sup> The present report deals with changes in the p.d. across the protoplasm when a cell is transferred from natural sea water to certain artificial solutions resembling sea water, in which the concentration of KCl is varied from 0 to 0.500 mol per liter.

These solutions had the following composition, based on a recipe for artificial sea water recommended by McClendon, Gault, and Mulholland:<sup>2</sup>

		Cl	0.570 molar
K + Na	0.500 molar	Br	0.001 "
Ca	0.011 "	SO <sub>4</sub>	0.028 "
Mg	0.054 "	HCO <sub>3</sub>	0.003 "

An artificial sea water made up in accordance with this formula, taking 0.012 as the molar concentration of KCl, has proved to be a satisfactory imitation of natural sea water for the purposes of these experiments. *Valonia* cells have been kept in this solution for weeks, and apparently will live in it indefinitely. The p.d. across the protoplasm of *Valonia* cells immersed in this solution is in good agreement with the values observed in natural sea water.

Measurements of E.M.F. were made by a compensation method, using a simple type of potentiometer and a Compton quadrant electrometer. By using this combination as a deflection potentiometer, as many as three readings per minute could be taken provided that the p.d. was not changing so rapidly as to require

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<sup>1</sup> For analyses of *Valonia* sap and a bibliography, see Osterhout, W. J. V., *Biol. Rev.*, 1931, 6, 155-216, particularly Table I, p. 158, and Footnotes 1 and 2, p. 156.

<sup>2</sup> McClendon, J. F., Gault, C. C., and Mulholland, S., *Carnegie Institution of Washington, Pub. No. 251*, 1917.

resetting the potentiometer. The sensitivity of the electrometer permitted reading to 0.1 mv.

The technic of measuring P.D. with *Valonia* has been described in detail in earlier papers.<sup>3</sup> The cell, supported by a four-pronged cork mount, is impaled on a glass capillary filled with artificial sap, through which electrical connection with the vacuole is established. Contact with the outside of the cell is made through a strip of wet filter paper touching its highest point; the solutions applied to the cell flow down the filter paper and over the entire cell surface. The rate of flow is usually 2 to 3 cc. per minute. When the solution applied to the cell is changed, the cell is rinsed with about 5 cc. of the new solution delivered rapidly from a pipet. This rapid rinsing is a small but important improvement in technic in cases where

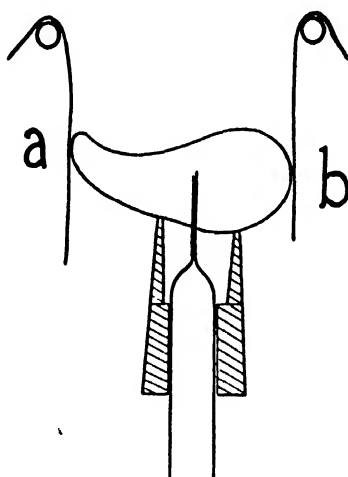


FIG. 1. Diagram showing the arrangement of an impaled *Valonia* cell (supported on a cork mount) in an experiment to demonstrate the effect of a second solution wetting a part of the cell surface.

the P.D. passes through a maximum. It has been shown<sup>3</sup> that the P.D. may be decreased considerably by the presence of a second solution wetting a part of the surface of the cell; consequently, too slow rinsing may prevent the P.D. from reaching its full maximum value.

The following experiment furnished a particularly striking example of the effect of a second solution wetting a part of the cell surface. The arrangement of the cell is shown in Fig. 1. Strips of wet filter paper, *a* and *b*, served to make electrical contact with opposite ends of a rather long cell, impaled with its long axis nearly horizontal. The P.D. measured directly between the ends of the cell could then be compared with the difference between the P.D.'s measured between

<sup>3</sup> Damon, E. B., *J. Gen. Physiol.*, 1929-30, **13**, 207.

the interior of the cell and the opposite ends. When natural sea water was applied at both ends, the P.D. between the interior of the cell (positive in the external circuit) and *a* was found to be 4.6 mv., that between the interior and *b*, 5.4 mv. The difference, 0.8 mv., was in good agreement with the value, 0.9 mv., measured directly between *a* (positive) and *b*. This is in accord with the behavior of intact cells, where the more pointed end, at which the cell had previously been joined to the parent cell, is generally found to be slightly positive with respect to the opposite end. When natural sea water at *a* was replaced by KCl-sea water (*i.e.*, the artificial sea water described above with all NaCl replaced by KCl), *b* became positive with respect to *a*, the P.D. varying between 3.8 and 5.8 mv. This agrees with the usual behavior of intact cells in such experiments. Measurement between the interior of the cell (positive) and KCl sea water at *a* showed 12.5 mv., between the interior and natural sea water at *b*, 7.1 mv., difference, 5.4 mv. KCl-sea water was then applied at both ends of the cell, the entire surface was rinsed with KCl-sea water, and the P.D. was measured between the interior of the cell and the two ends, *a* and *b*, connected together. This P.D. rose rapidly, passing through a maximum value of over 70 mv., which we shall see agrees with the usual behavior of impaled cells measured with KCl-sea water. From this and similar experiments we conclude (1) that these measurements were not affected by impalement,<sup>4</sup> and (2) that the P.D. with KCl-sea water may be greatly diminished if a part of the cell surface remains wet with natural sea water.

Electrical effects observed when *Valonia* cells are exposed to artificial sea waters containing different concentrations of KCl are illustrated by the P.D.-time curves, Fig. 2. These seven curves represent a series of measurements on the same cell using samples of modified artificial sea water in which the concentrations of KCl (in the order in which the measurements were carried out) were 0.400, 0.500, 0.300, 0.200, 0.100, 0.050, and 0.000 mol per liter. The pH of the solutions used in this series was adjusted to the same value as that of natural sea water, as indicated by the color of cresol red.

The P.D.-time curves with KCl-rich sea waters closely resembled the curves obtained with natural and artificial *Valonia* sap,<sup>5</sup> and are doubtlessly to be interpreted in the same way. In both cases there was an initial rise to a maximum, followed by a rapid fall to a minimum, and then a gradual rise to a second maximum. This behavior has

<sup>4</sup> In all experiments, the impaled cells are allowed to stand in sea water for at least 2 days before the first measurement, to permit the cell to form a good seal between the protoplasm and glass, and to recover from any temporary effects of impalement.

<sup>5</sup> Damon, E. B., *J. Gen. Physiol.*, 1931-32, **15**, 525.



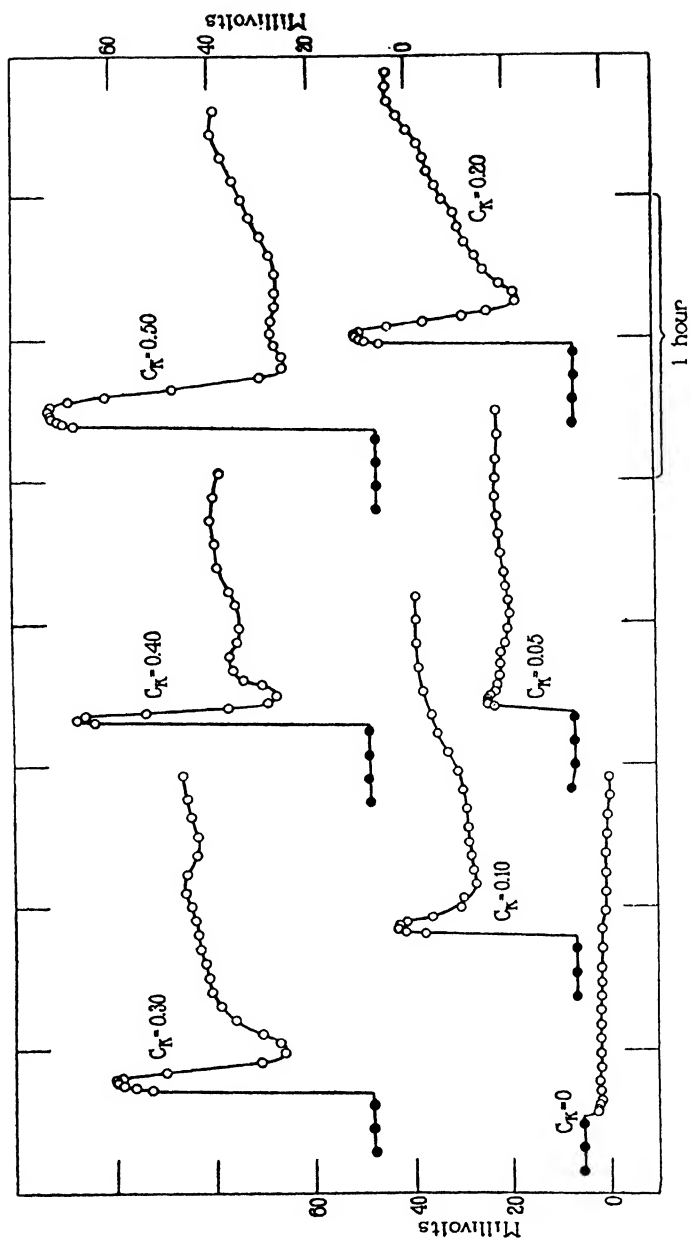


FIG. 2. P.D.-time curves, obtained from a series of measurements on the same *Valonia* cell, showing the changes in the P.D. across the protoplasm which are observed when the external solution is changed from natural sea water (shaded circles) to artificial modified sea waters (open circles) in which the concentration of KCl is varied from 0 to 0.500 mol per liter. The scale of ordinates at the left applies to the 4 lower curves; the scale at the right to the 3 upper curves. The sign is that of the inside of the cell; *i.e.*, for positive values of P.D., positive current tends to flow in an external circuit from the capillary through the measuring instrument to the solution applied externally.

been interpreted as due to an increase in the concentration of KCl in the main body of the protoplasm.<sup>3,5</sup> If this explanation is correct, the shape of the p.d.-time curves should serve as a rough measure for comparing the rates at which KCl enters the protoplasm under different conditions; *i.e.*, other things being equal, more rapid penetration of KCl should cause the curve to fall earlier and more sharply from its first maximum, and also to rise again more rapidly after passing through a minimum.

Application of this hypothesis to experiments in which the pH of the external solution was varied brings up some interesting questions. It has been found that the p.d. across the protoplasm of *Valonia* cells in natural sea water is not affected appreciably by changes in the pH within the range pH 5 to pH 10. We may therefore expect that within these limits changes in the pH of KCl-rich sea water will not alter the value of the initial maximum p.d. Certain theories<sup>6</sup> which have been proposed to account for the accumulation of KCl in *Valonia* sap, however, lead to the prediction that varying the pH of KCl-rich sea water applied to the cell should change the rate at which KCl enters the protoplasm, and hence should affect the shape of the p.d.-time curve. These theories, while differing in several important respects, are alike in connecting the mechanism of accumulation with the difference between the pH of the sap and that of the external sea water, a difference which is supposed to be maintained by the production of  $\text{H}_2\text{CO}_3$  or some other acid in the cell. Increasing this difference by raising the pH of the external solution should enhance the rate at which KCl enters the protoplasm; conversely, lowering the pH should inhibit the entrance of KCl.

These conclusions were tested by comparing the p.d.-time curves observed when a cell was exposed to samples of KCl-rich sea water of different pH. Effects due to variations in pH can be detected more easily with solutions containing a relatively low concentration of KCl, since with these solutions the characteristic fluctuations in p.d. are ordinarily not very rapid. In the artificial sea water used in this experiment the concentration of KCl was 0.050 molar. The pH of

<sup>6</sup> Briggs, G. E., *Proc. Roy. Soc. London, Series B*, 1930, **107**, 248. Brooks, S. C., *Protoplasma*, 1929, **8**, 389. Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1926, **24**, 234; *Biol. Rev.*, 1931, **6**, 155-216, particularly p. 199.

the more acid sample, determined by an indicator method, was about 5 at the beginning of the experiment; at the end, 3 days later, it had changed to about 6. The alkaline solution was initially at about pH

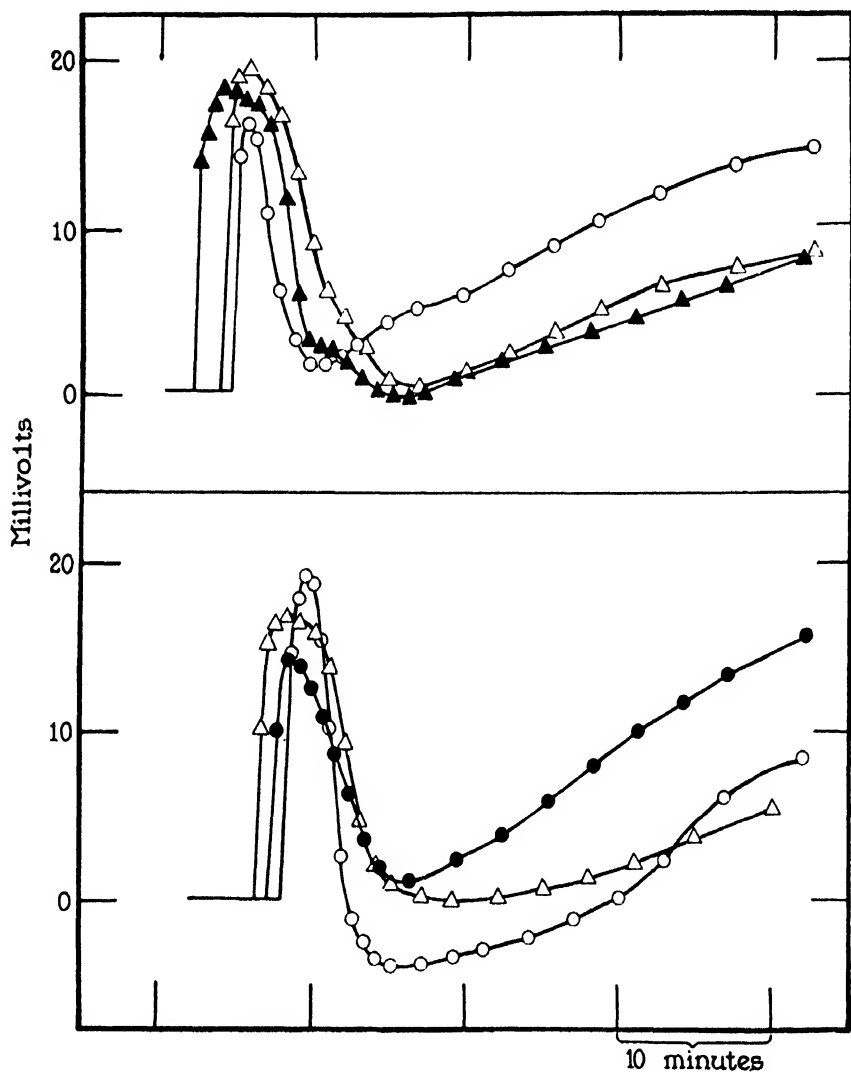


FIG. 3. P.D.-time curves, illustrating the effect of changing the pH of KCl-rich sea water containing 0.050 mol of KCl per liter from pH 5 (triangles) to pH 10 (circles). The shaded triangles or circles represent the last of the three measurements of each series. As ordinates are plotted the differences between the P.D. across the protoplasm in the KCl-rich sea water and the P.D. previously observed in natural sea water. The curves are displaced horizontally to prevent confusion.

10, as shown by incipient precipitation of  $\text{Mg}(\text{OH})_2$ , but probably became less alkaline before the end of the experiment. In order to distinguish between effects due to possible alteration in the cell during the experiment and effects actually produced by varying pH, three measurements were made on each cell, applying the acidified and alkaline solutions alternately. The cell was allowed to stand overnight in natural sea water between measurements. Results of such experiments with two different cells are shown in Fig. 3. It will be seen that in every case the first peak of the P.D.-time curve is broader in the measurements with the more acid solution, and that the rise after passing through a minimum is more moderate. This might be interpreted as evidence that KCl enters the protoplasm less rapidly from solutions of lower pH. To this extent, the experiment is in agreement with the theories for accumulation of KCl.

In the vacuolar sap, however (where the concentration of KCl is about 0.5 molar and the pH is about 6), the concentration of potassium is ten times as great as in the KCl-rich sea water here employed at pH 5, while the concentration of  $\text{H}^+$  is only about 1/10 as great. According to the theories for accumulation of KCl, we should expect potassium to come out of the cell under these conditions. The P.D.-time curves with this acidified KCl-rich sea water, on the contrary, have the characteristic shape which has been explained as due to the entrance of KCl. Furthermore, the P.D. in natural sea water remains constant when the pH is lowered to 5, showing no such changes as might be expected if KCl were coming out of the cell. This conflict between the theories dealing with the accumulation of KCl and the interpretation of these P.D.-time curves can probably be decided by a study of the composition of the sap of *Valonia* cells which have been exposed to acidified sea water.<sup>7</sup>

Since lowering the pH of KCl-rich sea water broadens the first peak of the P.D.-time curve without apparently affecting the value of the maximum P.D., the probability of observing the full value of this maximum can be increased by using acidified solutions. Advantage is taken of this in some of the later measurements described in this paper.

It is evident from inspection of Fig. 2 that the first maximum in

<sup>7</sup> Such experiments have been under way for some time at this laboratory, and will be published in the near future.

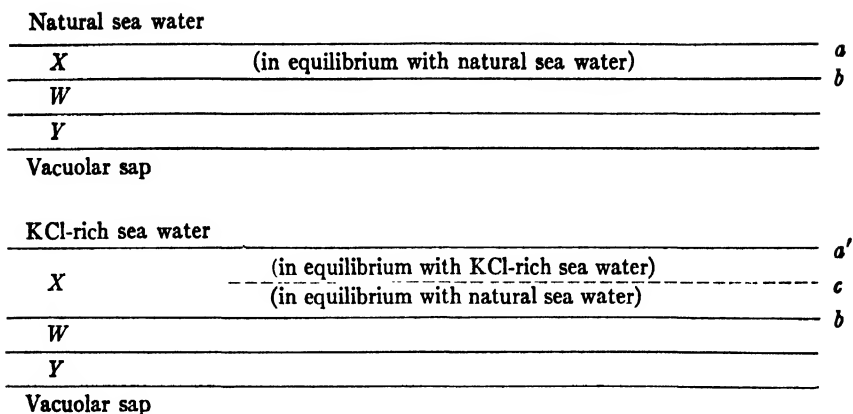


FIG. 4. Hypothetical diagrams illustrating the theory of protoplasmic layers. The middle layer, *W*, represents the main body of the protoplasm, assumed to be aqueous. *X* and *Y* represent the external and internal surface layers of the protoplasm, which are assumed to have the properties of two different non-aqueous liquids, immiscible with water. The observed P.D.'s are accordingly considered as made up of phase-boundary potentials at the surfaces of *X* and *Y*, plus diffusion potentials within the different layers.

The upper diagram represents conditions when the cell has been exposed to natural sea water for a long time, so that the external layer, *X*, is in distribution equilibrium with natural sea water. For the purposes of the present discussion, the P.D. at *b* (the inner surface of *X*) and all other P.D.'s located between *b* and the vacuolar sap may be lumped and called P.D.<sub>0</sub>. The P.D. with natural sea water may then be considered as the sum of P.D.<sub>0</sub> plus the phase-boundary potential at *a*:

$$P.D._{\text{natural s.w.}} = P.D._a + P.D._0$$

The lower diagram represents conditions supposed to correspond to the first maximum in the P.D.-time curve, soon after KCl-rich sea water has replaced natural sea water as the external solution. The outer region of the *X* layer, between *a'* and *c*, is now in distribution equilibrium with KCl-rich sea water, but between *c* and *b* concentrations still remain the same as when the cell was in natural sea water. At *b*, and below, everything remains unchanged; the value of P.D.<sub>0</sub> is therefore the same as in the measurement with natural sea water. At the outer surface of *X*, however, conditions have changed from the situation represented at *a* to that at *a'*, and at *c* a new diffusion potential has been set up. Hence we now have:

$$P.D._{\text{KCl-rich s.w.}} = P.D._{a'} + P.D._c + P.D._0$$

The change in P.D. across the protoplasm when natural sea water is replaced by KCl-rich sea water is accordingly:

$$P.D._{\text{KCl-rich s.w.}} - P.D._{\text{natural s.w.}} = P.D._{a'} + P.D._c - P.D._a$$

In other words, the initial rise in P.D. corresponds to the P.D. of the hypothetical chain:

KCl-rich sea water	region of the outer surface layer of the protoplasm in equilibrium with KCl-rich sea water	region of the outer surface layer of the protoplasm in equilibrium with natural sea water	natural sea water
$a'$	$c$	$a$	

the time curve represents the only value of the P.D. which can be correlated with the concentration of KCl in the external solution. From examination of these curves and other similar data it appears that greater regularity is found if we subtract from this value the P.D. previously observed in natural sea water. (Similarly, in studying the concentration effect with natural sea water<sup>8</sup> it was found that it is the difference between the P.D.'s in natural sea water and in diluted sea water which is proportional to the logarithm of the dilution.) In terms of the theory of protoplasmic layers,<sup>9</sup> illustrated in Fig. 4, we may assume that only the outer surface layer of the protoplasm, X, and the solutions applied externally are concerned in this first sharp rise in P.D. The difference between the initial maximum in the P.D.-time curve and the P.D. previously observed in natural sea water then represents the E.M.F. of the ideal system:

KCl-rich sea water	outer surface layer of the protoplasm	natural sea water
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In cases where this assumption is incorrect, some KCl having diffused through the external surface layer before the P.D. attains its maximum, the observed value will be lower than that corresponding to this ideal system. Other things being equal, therefore, the higher of two values

<sup>8</sup> Damon, E. B., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, **13**, 445.

<sup>9</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1927-28, **11**, 83. Osterhout, W. J. V., Damon, E. B., and Jacques, A. G., *J. Gen. Physiol.*, 1927-28, **11**, 193. Damon, E. B., *J. Gen. Physiol.*, 1929-30, **13**, 207; 1931-32, **15**, 525.

is probably the more reliable; extremely low values must be viewed with suspicion.

While the variations in the shape of the P.D.-time curves were less pronounced with KCl-rich sea water than with *Valonia* sap,<sup>3, 5</sup> the shape of the curve did not always permit assigning a definite value to the first maximum. In some cases the first peak was so sharp that the P.D. had begun to fall before the first measurement could be made. In other cases, the rise to the first maximum was so slow as to lead to suspicion that some KCl had diffused through the outer surface layer before the maximum P.D. was reached. This difficulty was generally found in cases where the cell had become coated with a gelatinous film of marine bacteria; it may have been caused by bacteria also in cases where this film was not detected. Occasionally the time curve passes through a point of inflection instead of a maximum;<sup>10</sup> in such cases the assignment of a definite value to the P.D. would obviously be more or less arbitrary.

In Fig. 5, differences between the P.D. in natural sea water and the first maximum in the P.D.-time curve with KCl-rich sea water are plotted against the concentration of KCl in the modified sea water. From a series of thirty-five measurements (including the ones reported in Fig. 2) which had been carried out primarily to study the form of the P.D.-time curve, twenty-nine curves were obtained which permitted assigning definite values to the first maxima. These values are represented by shaded circles in Fig. 5. The five points represented by triangles were taken from some earlier exploratory measurements, and may not be strictly comparable with the more recent data, since with these solutions, as with *Valonia* sap,<sup>5</sup> the P.D. may be considerably affected by the condition of the cells. Measurements with KCl-free sea water were not included in Fig. 5 because this solution seemed to be injurious to *Valonia*. While in some experiments the behavior of KCl-free sea water agreed with the curve marked " $C_K = 0$ " in Fig. 2, in others secondary changes occurred similar to those observed when *Valonia* cells are exposed to hypotonic diluted sea water for a short time, or to isotonic diluted sea water for a longer time.<sup>8</sup> These

<sup>10</sup> A P.D.-time curve of this form (with artificial sap) is shown in an earlier report, *J. Gen. Physiol.*, 1929-30, **13**, 215, Fig. 6, Curve B.

changes are evidenced by erratic fluctuations in the P.D., and sometimes by high P.D.'s of opposite sign (inside of the cell negative). It now appears that a moderate reduction in the concentration of KCl does not necessarily involve injury to the cell, since Jacques and

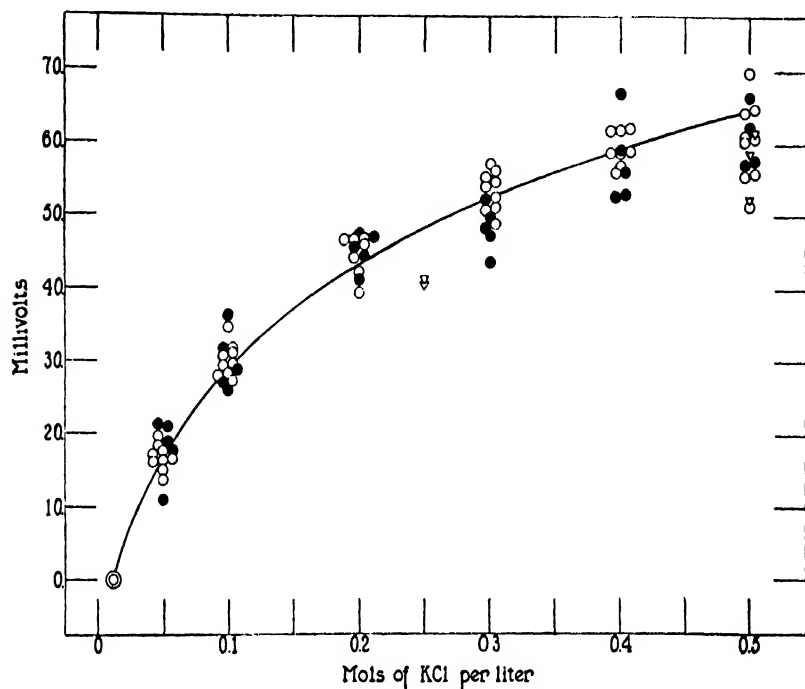


FIG. 5. Curve showing the relation between the concentration of KCl in modified sea water and the P.D. across the protoplasm in *Valonia*. The ordinates represent differences between the P.D. in natural sea water and the first maximum in the P.D.-time curve when natural sea water was replaced by KCl-rich sea water. Each group of points represents measurements with a single concentration of KCl but in order to show all the observed values some of the points have been displaced to the right or left of their true abscissae.

Triangles represent five measurements on four *Valonia* cells, collected Nov. 25, 1929, measured Jan. to Feb., 1930. Shaded circles represent twenty-nine measurements on fifteen different cells, collected Nov. 14, 1930, measured Nov., 1930, to Jan., 1931. In these measurements the modified sea water had the same pH as natural sea water. Open circles represent fifty-one measurements on ten individuals, collected May 25, 1931, measured June to July, 1931. In these measurements, the modified sea water was acidified to pH 6.

The curve drawn through these groups of points is identical with the curve drawn in Fig. 6.



Osterhout<sup>11</sup> have recently found that *Valonia* cells survived exposure for 20 days to modified sea water containing one half the usual amount of KCl.

While these data with KCl-rich sea water showed that the initial change in P.D. varies in a regular manner with the concentration of KCl, they seemed hardly adequate for determining the exact relation between P.D. and concentration. Since these thirty-four accepted values represented measurements on nineteen different cells, an average of less than two measurements per cell, possible variations among the individual cells might well prove very misleading. Furthermore, the rejection of a number of unsatisfactory observations introduced an undesirable subjective factor. Accordingly, it seemed worth while to carry out a new series of measurements for the express purpose of determining the value of the initial change in P.D. when sea water is replaced by KCl-rich sea water.

In the earlier experiments, the cells had been exposed to modified sea water for an hour or more in each measurement; the solutions had been adjusted to the same pH as natural sea water. In the new series, the pH of the KCl-rich sea waters was lowered to 6 in order to broaden the first peak of the P.D.-time curves, and thus increase the probability of observing the full value of the P.D. change. To reduce to a minimum any possible alteration in the cell produced by exposure to KCl-rich solutions, the cells were left in contact with these solutions only just long enough to determine the full value of the P.D. change; as soon as the P.D. had started to fall from its first peak (generally within about 3 minutes) the cells were replaced in natural sea water and allowed to stand overnight or longer before the next measurement. No evidence of marine bacteria in quantities which could be detected by macroscopic observation was noted during these experiments. The data obtained in this series, fifty-one measurements on ten cells, are shown plotted as open circles in Fig. 5. In a number of cases, where the cell was measured more than once with a given solution, the highest observed value of the P.D. is reported. The agreement of this series with the earlier data confirms the assumption that the values of the first maxima are not altered by moderate changes in the pH of KCl-rich sea water.

<sup>11</sup> Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1931-32, **15**, 537.

The new data are free from some of the defects of the earlier series. Due to the larger number of measurements with each cell, variations among the individual cells affect all parts of the P.D.-concentration curve more or less equally. Since doubtful measurements could be repeated, it proved unnecessary to reject any measurement except in favor of a similar measurement with the same cell. The personal factor was thus greatly decreased, although not wholly eliminated. Another uncertainty still remained: it became apparent that the behavior of some of the cells was changing considerably during the series of measurements. It was difficult to estimate how nearly such changes in different cells would compensate one another.

It was concluded that the most reliable data for determining the relation between P.D. and concentration of KCl would consist in a series of measurements in duplicate on a single cell, in which the agreement between the first and second values observed with each solution would indicate to what extent the cell had changed during the experiment. Several series of measurements in duplicate were carried out in accordance with this plan. The most successful of these, in which the cell evidently suffered no significant alteration, is recorded in Fig. 6 (upper curve).

In accordance with the theory of protoplasmic layers<sup>9</sup> illustrated in Fig. 4, the P.D.'s plotted in Fig. 5 and Fig. 6 may be interpreted as equivalent to the P.D. of the ideal system:

KCl-rich sea water	region of the outer surface layer of the protoplasm in equilibrium with KCl-rich sea water	region of the outer surface layer of the protoplasm in equilibrium with natural sea water	natural sea water
$a'$	$c$	$a$	

where the outer surface layer of the protoplasm is assumed to have the properties of a non-aqueous liquid immiscible with water. According to various theories which have been proposed for calculating such P.D.'s, the observed value may be made up of (1) phase-boundary potentials at  $a$  and  $a'$  and (2) a diffusion potential in the non-aqueous layer at  $c$ . Since the calculation of such mixed potentials is a formida-

ble task, the assumption is often made that the relative mobilities of ions in the non-aqueous layer are not greatly different from their values in aqueous solutions, and hence that the diffusion potential at  $c$  will

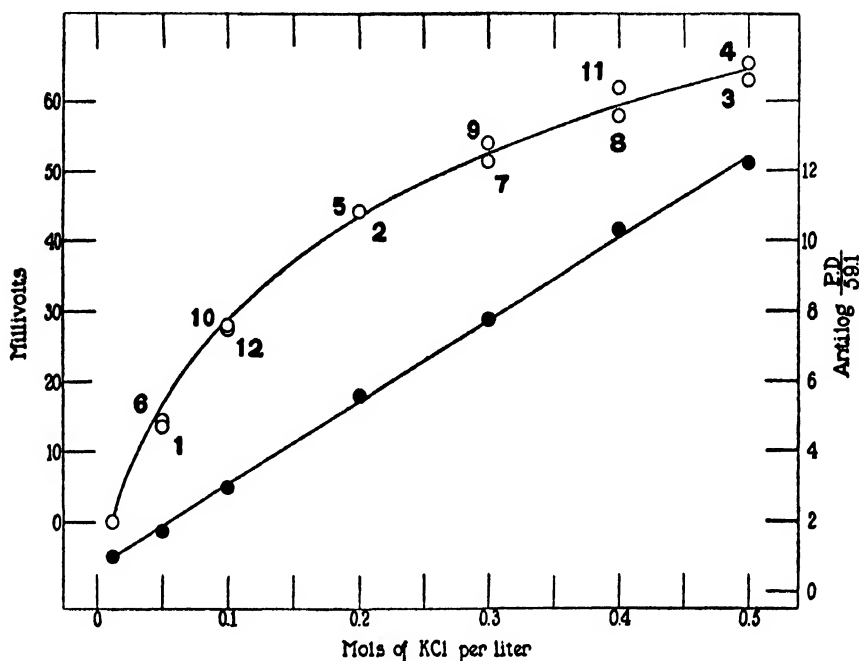


FIG. 6. Curves showing the relation between the concentrations of KCl in modified sea water (plotted as abscissae) and the P.D. across the protoplasm in *Valonia*.

The ordinates of the upper curve (scale at left) represent differences between the P.D. in natural sea water and the first maximum in the P.D.-time curve when natural sea water was replaced by KCl-rich sea water. The data represented by open circles were obtained in a series of measurements in duplicate using the same *Valonia* cell, the numbers adjacent to these circles indicating the order in which the measurements were made. Measurements were at room temperature, which varied between 23.5° and 26.5°, average, 25°C. The *Valonia* cell was collected May 25, 1931, measured June 16 to July 3, 1931.

The ordinates of the lower curve (scale at the right) represent the antilogarithms of the quotients of these P.D. changes divided by  $\frac{RT}{0.434 F}$  (which at 25°C. has the value 59.1 mv.). The values plotted as shaded circles were calculated from the averages of the two P.D.'s plotted as open circles directly above. The upper curve was obtained from the straight line drawn through these shaded circles: each ordinate of the upper curve is equal to 59.1 multiplied by the logarithm of the corresponding ordinate of the straight line.

be negligibly small as compared with the phase-boundary potentials at  $a$  and  $a'$ . If so, phase-boundary potentials calculated by assuming suitable values for the hypothetical ionic partition coefficients should agree with the observed P.D.'s. But since these ionic partition coefficients and the mobilities in the non-aqueous layer are alike unknown, it is just as reasonable to assume that the partition coefficients of the various ions are approximately equal, in which case the phase-boundary potentials at  $a$  and  $a'$  will be negligibly small. The observed P.D. would then represent only the diffusion potential at  $c$ . It is interesting to compare the observed P.D.'s with the values calculated in accordance with each of these mutually contradictory assumptions.

The formula for calculating phase-boundary potentials with mixed electrolytes has been derived by Michaelis and Fujita<sup>12</sup> and by Horovitz.<sup>13</sup> According to their equation, the P.D. at either phase-boundary,  $a$  or  $a'$ , is given by the expression:

$$(1) \quad \text{P.D.} = \frac{RT}{2F} \ln \frac{A_K C_K + A_{Na} C_{Na}}{A_{Cl} C_{Cl}}$$

where  $C_K$ ,  $C_{Na}$ , and  $C_{Cl}$  represent the concentrations<sup>14</sup> of these ions in the natural or modified sea water, and  $A_K$ ,  $A_{Na}$ , and  $A_{Cl}$  the "true" ionic partition coefficients. The Ca, Mg, and  $\text{SO}_4$  ions are omitted because it has been shown that P.D.'s observed with diluted sea water are not affected by considerable changes in the concentrations of these ions.<sup>8</sup> It may therefore be assumed that the partition coefficients of these ions are small (an assumption which is in agreement with the low concentrations of these ions found in the vacuolar sap) and hence that the products,  $A \cdot C$ , for these ions may be neglected in comparison with the corresponding products for K, Na, and Cl. Other ions are not considered because their concentrations in sea water are very small. It has been shown, for example, that the P.D. is independent of rather large changes in pH. The P.D. between natural sea water and KCl-

<sup>12</sup> Michaelis, L., and Fujita, A., *Z. phys. Chem.*, 1924, **110**, 266.

<sup>13</sup> Horovitz, K., *Z. phys. Chem.*, 1925, **115**, 424.

<sup>14</sup> For the sake of simplicity, concentrations have been used instead of activities in this discussion. Since the natural and modified artificial sea waters used in these measurements were all solutions of the same ionic strength, and since the activity coefficients for KCl and NaCl at this ionic strength are not very different, errors introduced by omitting the activity coefficients should be small, and approximately the same in all cases.

rich sea water, the algebraic sum of the P.D.'s at  $a$  and  $a'$ , will then be given by the following expression:

$$(2) \quad \text{P.D.} = \frac{RT}{2F} \ln \frac{A_K C'_K + A_{Na} C'_{Na}}{A_{Cl} C'_{Cl}} + \frac{RT}{2F} \ln \frac{A_{Cl} C_{Cl}}{A_K C_K + A_{Na} C_{Na}}$$

where primes indicate concentrations in the KCl-rich sea water. Since the concentration of Cl is the same on both sides, the equation becomes:

$$(3) \quad \text{P.D.} = \frac{RT}{2F} \ln \frac{A_K C'_K + A_{Na} C'_{Na}}{A_K C_K + A_{Na} C_{Na}}$$

It can be shown, however, that this equation is inadequate to account for the P.D. changes observed with *Valonia*. For example, we may consider the P.D. between natural sea water and KCl-sea water (in which all the NaCl is replaced by KCl) for which the observed values plotted in Fig. 5 range between 51.4 and 69.9 mv. If we insert numerical values in Equation 3:

$$\text{P.D. (in mv.)} = \frac{59.1}{2} \log \frac{0.500 A_K}{0.012 A_K + 0.488 A_{Na}}$$

and then divide the numerator and denominator of the fraction by  $0.500 A_K$  we get:

$$\text{P.D.} = 29.6 \log \frac{1}{0.024 + 0.976 \frac{A_{Na}}{A_K}}$$

We may now calculate the P.D. according to this equation by assuming different values for  $A_K$ . If we try first the assumption that  $A_K$  may be 100 times as great as  $A_{Na}$ , the calculated P.D. is found to be 43.5 mv., which, although of the right order of magnitude, is obviously too low. Materially better agreement with the observed values cannot be obtained, however, by assuming a larger value for  $A_K$ , since if  $A_K$  is allowed to increase without limit we find that the P.D. approaches the limiting value of only 47 mv. It is obvious that no positive value which can be assigned to  $A_K$  will account for the observed potentials.

We may now test whether the P.D. with KCl-rich sea water can be calculated more satisfactorily as a diffusion potential (at  $c$ ) by assuming a reasonable value for the mobility of  $K^+$  in the non-aqueous layer. This calculation is greatly simplified by the assumption that the ionic partition coefficients,  $A_K$  and  $A_{Na}$ , are equal, and hence that the actual partition coefficients of KCl and NaCl are equal. It follows from this assumption that the sum of the phase-boundary potentials at  $a$  and  $a'$  is equal to zero.

In this calculation only  $K^+$ ,  $Na^+$ , and  $Cl^-$  are considered; other ions are neglected for the same reasons as in the above calculation of phase-boundary potentials. Either Planck's or Henderson's formula for the liquid junction potential may be taken as a starting point, since both reduce to the same expression for the case of two electrolytes with common anion at the same concentration:

$$(4) \quad \text{P.D.} = \frac{RT}{F} \ln \frac{c'_{Na}u_{Na} + c'_K u_K + c_{Cl}v_{Cl}}{c_{Na}u_{Na} + c_K u_K + c_{Cl}v_{Cl}}$$

where  $u_K$ ,  $u_{Na}$ , and  $v_{Cl}$  represent the mobilities of these ions in the non-aqueous layer,  $c_{Na}$ ,  $c_K$ , and  $c_{Cl}$  the concentrations of these ions in the non-aqueous layer in equilibrium with natural sea water, and  $c'_{Na}$ ,  $c'_K$ , and  $c_{Cl}$  their concentrations in the non-aqueous layer in equilibrium with KCl-rich sea water. (The concentration of Cl is the same on both sides.) In accordance with our assumption that the partition coefficients of  $K^+$  and  $Na^+$  are equal ( $A_K = A_{Na} = A$ ) we may express the concentrations in the non-aqueous layer in terms of the concentrations in the sea waters:

$$\begin{aligned} c_{Na} &= A \cdot C_{Na} & c'_{Na} &= A \cdot C'_{Na} & c_K &= A \cdot C_K & c'_K &= A \cdot C'_K \\ c_{Cl} &= A (C_{Na} + C_K) & & & & & &= A (C'_{Na} + C'_K). \end{aligned}$$

Substituting in Equation 4:

$$\begin{aligned} \text{P.D.} &= \frac{RT}{F} \ln \frac{AC'_{Na}u_{Na} + AC'_K u_K + A(C_{Na} + C_K) v_{Cl}}{AC_{Na}u_{Na} + AC_K u_K + A(C_{Na} + C_K) v_{Cl}} \\ (5) \quad &= \frac{RT}{F} \ln \frac{C'_{Na}u_{Na} + C'_K u_K + (C_{Na} + C_K) v_{Cl}}{C_{Na}u_{Na} + C_K u_K + (C_{Na} + C_K) v_{Cl}}. \end{aligned}$$

We may now substitute in Equation 5 certain numerical values: the concentrations of Na and K in natural sea water, and the value of  $RT/F$  at 25°C.; at the same time we may change from natural to common logarithms:

$$(6) \quad \text{P.D. (in mv.)} = 59.1 \log \frac{C'_{\text{Na}} u_{\text{Na}} + C'_{\text{K}} u_{\text{K}} + 0.500 v_{\text{Cl}}}{0.488 u_{\text{Na}} + 0.012 u_{\text{K}} + 0.500 v_{\text{Cl}}}$$

The agreement of the data with an equation of this type can be tested more readily if Equation 6 is rearranged (using the relation,  $C'_{\text{Na}} + C'_{\text{K}} = 0.500$ ) into:

$$(7) \quad \text{antilog} \frac{\text{P.D.}}{59.1} = \frac{u_{\text{K}} - u_{\text{Na}}}{0.488 u_{\text{Na}} + 0.012 u_{\text{K}} + 0.500 v_{\text{Cl}}} C'_{\text{K}} + \frac{0.500 (u_{\text{Na}} + v_{\text{Cl}})}{0.488 u_{\text{Na}} + 0.012 u_{\text{K}} + 0.500 v_{\text{Cl}}}$$

which is an equation of the form:

$$\text{antilog} \frac{\text{P.D.}}{59.1} = B C'_{\text{K}} + D$$

where  $B$  and  $D$  are constants. Accordingly, if values of  $\text{antilog} \frac{\text{P.D.}}{59.1}$  calculated from observed values of P.D. are plotted as ordinates against the corresponding concentrations of KCl in modified sea water as abscissae, the points should fall along a straight line.

In Fig. 6, averages of the P.D. values represented by open circles in the upper curve were used in computing the values of  $\text{antilog} \frac{\text{P.D.}}{59.1}$  plotted as shaded circles directly below. It is evident that the points represented by these shaded circles do adhere very closely to the straight line which has been drawn through them. The curve drawn through the open circles in Fig. 6 was obtained from this straight line; *i.e.*, each ordinate of the upper curve is equal to 59.1 multiplied by the logarithm of the corresponding ordinate of the straight line. This upper curve therefore corresponds to Equation 6.

We conclude, therefore, that the first rise in the P.D.-time curve when natural sea water is replaced by KCl-rich sea water may be

calculated satisfactorily by substituting in Equation 6 suitable values for the relative mobilities of  $K^+$ ,  $Na^+$ , and  $Cl^-$  in the non-aqueous layer. From measurements of the concentration effect with natural sea water<sup>3</sup> the mobility of  $Cl^-$  in *Valonia* protoplasm has been found to be five times as great as that of  $Na^+$ . While the absolute values of these mobilities are of course unknown, in these calculations we may equally well use relative mobilities referred to the mobility of  $Cl^-$  taken as unity; i.e.,  $v_{Cl} = 1.00$ ,  $u_{Na} = 0.20$ . We may calculate the relative mobility of  $K^+$  by inserting these values in Equation 6 or 7. For the case of the cell used in the measurements reported in Fig. 6, the relative mobility of  $K^+$  is found to be 20. From the spread of the P.D. values plotted in Fig. 5, it is apparent that there is a considerable variation in the value of  $u_K$  among different cells. Evidently the value of  $u_K$  may also vary considerably in the same individual under different conditions.<sup>15</sup> Since the curve drawn through the groups of points in Fig. 5 is identical with the curve drawn in Fig. 6, it is evident that the value,  $u_K = 20$ , is in good agreement with the average behavior of all the cells included in this report.

While it has been shown that the observed values of P.D. with KCl-rich sea water are accurately reproduced by Equation 6, it does not

<sup>15</sup> The relative mobility of  $Na^+$  may also vary, but the good agreement usually found among measurements of concentration effect with natural sea water suggests that  $u_{Na}$  is subject to less variation than  $u_K$  in cells of similar history. *Valonia* cells which have been kept at the laboratory for a long time, especially cells which have received relatively little illumination, show certain differences from recently collected cells. Observations made at different times using "old" cells with different histories (for which reason the data may not be strictly comparable) indicate that with such "old" cells (1) the P.D. with KCl-rich solutions is lower, and hence the value of  $u_K$  is smaller; (2) the concentration effect with natural sea water is smaller, and hence the value of  $u_{Na}$  is larger; (3) the ratio of  $K \div Na$  in the sap is lower than in recently collected cells. It is hoped that this apparent correlation between the composition of the sap and the relative mobilities of  $K^+$  and  $Na^+$  can be investigated more carefully.

Dr. L. R. Blinks has suggested (private communication) that the apparent variation in  $u_K$  from cell to cell may actually represent the variation of some other factor, such as the thickness of cell wall or of protoplasm, which would affect the speed with which concentration changes reach  $W$ , and hence the height of the maximum in the P.D.-time curve. The highest calculated value of  $u_K$  should accordingly be regarded as a minimum value for the relative mobility of  $K^+$ .



necessarily follow that the assumptions used in deriving this equation are correct. Any equation of the same general form, *i.e.*

$$\text{P.D.} = \frac{RT}{F} \ln (B C'_K + D)$$

will of course fit the data equally well. It is possible that an equation of this type might be derived on the basis of entirely different assumptions, in which case the constants of the equation would be interpreted differently. Since, however, the equation has actually been derived on the assumption that the observed P.D. represents a diffusion potential, it is convenient to refer to the constants,  $u_K$ ,  $u_{Na}$ , and  $v_{Cl}$ , as apparent relative mobilities. It is hoped that these values, and the apparent relative mobilities of other ions obtained in the same way, may prove useful in interpreting bioelectric measurements with *Valonia*.

#### SUMMARY

The P.D. across the protoplasm of *Valonia macrophysa* has been studied while the cells were exposed to artificial solutions resembling sea water in which the concentration of KCl was varied from 0 to 0.500 mol per liter. The P.D. across the protoplasm is decreased by lowering and increased by raising the concentration of KCl in the external solution. Changes in P.D. with time when the cell is treated with KCl-rich sea water resemble those observed with cells exposed to *Valonia* sap.

Varying the reaction of natural sea water from pH 5 to pH 10 has no appreciable effect on the P.D. across *Valonia* protoplasm. Similarly, varying the pH of KCl-rich sea water within these limits does not alter the height of the first maximum in the P.D.-time curve. The subsequent behavior of the P.D., however, is considerably affected by the pH of the KCl-rich sea water. These changes in the shape of the P.D.-time curve have been interpreted as indicating that potassium enters *Valonia* protoplasm more rapidly from alkaline than from acidified KCl-rich sea water. This conclusion is discussed in relation to certain theories which have been proposed to explain the accumulation of KCl in *Valonia* sap.

The initial rise in P.D. when a *Valonia* cell is transferred from natural sea water to KCl-rich sea water has been correlated with the concen-

trations of KCl in the sea waters. It is assumed that the observed p.d. change represents a diffusion potential in the external surface layer of the protoplasm, where the relative mobilities of ions may be supposed to differ greatly from their values in water. Starting with either Planck's or Henderson's formula, an equation has been derived which expresses satisfactorily the observed relationship between p.d. change and concentration of KCl. The constants of this equation are interpreted as the relative mobilities of  $K^+$ ,  $Na^+$ , and  $Cl^-$  in the outer surface layer of the protoplasm. The apparent relative mobility of  $K^+$  has been calculated by inserting in this equation the values for the relative mobilities of  $Na^+$  (0.20) and  $Cl^-$  (1.00) determined from earlier measurements of concentration effect with natural sea water. The average value for the relative mobility of  $K^+$  is found to be about 20. The relative mobility may vary considerably among different individual cells, and sometimes also in the same individual under different conditions.

Calculation of the observed p.d. changes as phase-boundary potentials proved unsatisfactory.



# THE ACTION OF X-RAYS ON EUPLOTES TAYLORI AND ASSOCIATED BACTERIA\*

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In the first two papers of this series (1, 2) the results of preliminary investigations on the rôle of bacteria in the nutrition of protozoa were reported. Apart from demonstrating a curious dependence of *Euplotes taylori* upon two strains of living bacteria these introductory studies centered around the problem of obtaining bacteria-free protozoa. Two methods of sterilization were examined in detail, and shown to be experimentally feasible,—repeated immersion in sterile media, and the killing of bacteria with appropriate bactericidal agents. Through the use of soft x-rays we have now been able to develop a third method of considerable promise, which, along with related observations on the lethal action of x-rays, we propose to discuss in this communication.

## Apparatus

The x-ray equipment used in these investigations was designed by Dr. Harry Clark. High ionization intensity has been obtained by a short target-to-specimen distance (3 cm.), silver anode, thin aluminum window (0.003 inches) and moderately high power (30 ma. at 50 kv.). The intensity as measured by an ionization chamber was maintained at 2110 Roentgen units per second in the experiments of Part I and at 2530 Roentgen units per second in the later studies.

## PART I

### *The Action of X-rays on Euplotes taylori*

Throughout the investigation we employed thriving cultures of *Euplotes taylori* which had been maintained for many generations in

\* Contribution No. 3 of "Studies on Protozoa," supported by a grant from the Committee on Effect of Radiation on Living Organisms, National Research Council.

1:1 artificial sea water on a mixture of two strains of bacteria (*Pseudomonas fluorescens* and *Bacillus coli*.  $K_{13}$ )<sup>1</sup> in approximately equal proportions. 48 hours after the last feeding the protozoan culture was divided into two portions, the first for irradiation of the organisms in the motile state and the second for the preparation and irradiation of the encysted forms. For this purpose the organisms were caused to encyst by gradual evaporation of the medium from a partly open watch-glass at room temperature. Encystment was complete within 15 hours. Fifteen to forty-five of the organisms or cysts suspended in approximately 0.01 cc. of medium<sup>2</sup> were transferred to a sterile, shallow, spherically ground slide. This was provided with a moist chamber consisting of a circular trough ground into the concave depression near the edge, into which water could be placed. The whole was sealed with a sterile cover-slip of mica<sup>3</sup> held in position with vaseline. The slide was then inserted in the holder and irradiated for a stated time as indicated in Table I. The organisms were observed immediately after irradiation, 15 minutes later, and finally after 5 hours. In the case of the motile forms, special attention was given to any abnormalities in the movements of the organism (cessation of motion, spiral movements, marked changes in rate of movement), vacuolation, and disintegration. In many of the experiments the organisms were inactive and seemingly dead 15 minutes after irradiation but were found actively swimming at the end of 5 hours or later. In the case of the cysts attention was given to vacuolation and the rate of encystment. As a rule those cysts with many vacuoles were found to be dead. Marked vacuolation and failure to excyst in 1:1 artificial sea water were accepted as evidence of death.

<sup>1</sup> For a description of the organisms and culture medium, cf. the first paper of this series (1). Organism A, mentioned therein, has since been identified as *Pseudomonas fluorescens*.

<sup>2</sup> The motile organisms were suspended in 1:1 artificial sea water, and the cysts in 1:1 artificial sea water which by evaporation had been reduced to 1/20 of its initial volume.

<sup>3</sup> It was necessary to measure accurately the thickness of all mica cover-slips and to employ correction factors for variations therein. Mr. Hugo of the Stanford Gauge Laboratory, was good enough to make the measurements. In preliminary experiments where the slips were only approximately similar, appreciable differences in absorption were observed.

On completion of the 5 hour microscopic examination, ten to twenty of the organisms,<sup>4</sup> either as cysts or in the motile state, were transferred to 1:1 artificial sea water and fed with the appropriate bacteria. On the 2nd day the numbers of actively motile and apparently normal organisms were counted, the values so obtained serving as an index of viability. These results together with intermediate counts made upon the motile forms are entered in Table I.

Finally, sterility tests upon the irradiated organisms were also run. For this purpose the entire contents of the slide, after removal of ten to twenty organisms for the viability test, were transferred to nutrient agar. We were interested to observe that brief periods of irradiation, even of 10 to 30 seconds, were sufficient to sterilize the protozoa. It is this observation which has permitted us to prepare bacteria-free *Euplotes* with comparative ease. Although the ciliate suffers no immediately recognizable injury when irradiated for brief periods, there is evidence which will be presented later in the paper, that irradiation for 60 seconds materially reduces its longevity and power of continued reproduction.

It should be pointed out that all experiments were rigidly controlled by the use of parallel cultures of motile forms and cysts which were transferred to depression slides, subjected to microscopic examinations, and ultimately run through the viability and sterility tests. They differed from the experimental ones only in not being irradiated. In the sterility tests run upon these controls, numerous colonies appeared in the nutrient agar within a day or two.

The results of the experiments are presented in Table I.

Examination of the table indicates that 190 seconds of irradiation were sufficient to kill the encysted organisms. The motile forms required exposure for 220 seconds. Due to the different conditions of irradiation (*cf.* footnote 2) we are not disposed to conclude that these values are indicative of any fundamental difference in the resistance of encysted and excysted forms to death by x-rays. To investigate this possibility it will be necessary, presumably, to suspend the organisms in identical media and to maintain the bacterial content

<sup>4</sup> It should be mentioned that some of these organisms at the time of transfer were inactive and, in some cases, seemingly dead due to the curious effect of x-rays in bringing about a temporary suspension of ciliary movement.

TABLE I

*The Action of X-Rays on Euplotes taylora*

Time of irradiation	State	No. of experiments	Total No. of organisms irradiated	No. of organisms active* after irradiation Hours after exposure				Sterility of irradiated <i>Euplotes</i> . No. of pour-plate colonies
				0	½	5	48†	
<i>sec.</i>								
0	Cysts	5	147				272	Many
0	Motile	8	219				377	Many
10-30	Motile	6	199	199	199	197	375	0
30-45	Motile	7	218	218	218	220	373	0
60	Motile	5	152	152	152	152	242	Few
76-90	Motile	4	133	133	133	133	233	0
91-105	Cysts	6	201				218	0
91-105	Motile	7	189	177	156	171	264	0
106-120	Cysts	6	206				211	0
106-120	Motile	7	173	161	129	142	223	0
130	Cysts	5	153				188	0
130	Motile	3	78	67	56	55	96	Few
135-150	Cysts	16	495				156	0
135-150	Motile	15	376	317	184	214	416	1
151-165	Cysts	4	102				72	0
151-165	Motile	5	136	100	95	118	132	Few
166-180	Cysts	6	174				40	0
166-180	Motile	7	169	128	7	54	145	5
190	Cysts	2	55				0	0
190	Motile	2	34	6	1	5	24	0
196-210	Cysts	4	118				0	0
196-210	Motile	5	185	84	1	8	70	2
220	Cysts	6	171				0	0
220	Motile	5	127	0	0	0	0	0
230	Cysts	1	31				0	0
230	Motile	1	16	0	0	0	0	4
240	Cysts	3	89				0	0
240	Motile	4	116	0	0	0	0	0
260-270	Cysts	3	92				0	0
260-270	Motile	3	91	0	0	0	0	0

\* Ciliary activity and swimming.

† 43 hours earlier, a known number of the organisms,<sup>4</sup> approximately one-half, had been placed in 1:1 artificial sea water and fed with the appropriate bacteria. The values in this column are calculated for the entire number actually irradiated.

approximately constant. Other experiments which we have in progress indicate that the presence of much bacterial substance decreases the toxicity of x-rays towards protozoa.

Accepting the higher value of 220 seconds as the lethal exposure time for *Euplotes*, calculations may be made of the energy required to kill a single organism. If it be assumed that the absorption coefficient of cytoplasm is the same as that of water the amount of radiation absorbed in 220 seconds by 1 cc. of irradiated substance would be  $220 \times 2110$  Roentgen units. This equals  $4.6 \times 10^7$  ergs ( $220 \times 2110 \times 6.1 \times 10^{13} \times 1.591 \times 10^{-12}$ ). From the values published by Garnjobst (3) for the linear dimensions of *Euplotes taylori* its volume may be calculated as approximately  $2.1 \times 10^{-7}$  cc. The energy required to kill a single protozoon by the use of x-rays is therefore 9.7 ergs. This calculation assumes that radiation absorbed by the surrounding medium is not productive of toxic or protective substances, and that the absorption coefficient of cytoplasm equals that of water. The former assumption is of doubtful validity.

It is also clear from the table that all bacteria associated with *Euplotes* were killed with but a few seconds of exposure. This marked difference in the resistance of the protozoa and bacteria to death by x-rays suggested that similar differences might exist between the two species of bacteria. This possibility led us to the experiments reported in Part II.

## PART II

### *The Action of X-Rays on Pseudomonas fluorescens and Bacillus coli, K<sub>13</sub>*

The purpose of this inquiry was to determine the lethal dose of x-rays for the two strains of bacteria used as nutrients in the cultivation of *Euplotes taylori*. Approximately 0.01 cc. of a suspension of the appropriate organism, washed from an agar slant, was transferred to a depression slide, covered with mica, and irradiated for some stated time. 1:1 artificial sea water was used as the suspension medium. After irradiation the contents were transferred quantitatively to nutrient broth and tested for sterility. The results are presented in Table II. From these it may be concluded that approximately 15



seconds of exposure ( $3.8 \times 10^4$  Roentgen units) are sufficient to kill *Pseudomonas fluorescens*. *Bacillus coli*,  $K_{13}$ , requires approximately 45 seconds ( $9.4 \times 10^4$  Roentgen units). If we consider  $0.4\mu \times 1.4\mu$  and  $0.5\mu \times 1.5\mu$  to be the linear dimensions of *Pseudomonas fluorescens* and *Bacillus coli* respectively, and  $3.7 \times 10^6$  ergs to be the energy per cc. of the absorbed radiation it follows that  $5.9 \times 10^{-7}$  and  $29 \times 10^{-7}$  ergs represent the lethal energy per organism for *Pseudomonas fluorescens* and *Bacillus coli*, respectively. These values may be

TABLE II

*Lethal Dose of X-Rays for Pseudomonas fluorescens and Bacillus coli, K<sub>13</sub>*

Time groups <i>sec.</i>	<i>Pseudomonas fluorescens</i>			<i>Bacillus coli, K<sub>13</sub></i>		
	No. of slides	Viable	Sterile	No. of slides	Viable	Sterile
2.5-7.5	4	4	0	4	4	0
7.5-12.5	10	7	3	4	4	0
12.5-17.5	14	0	14	5	5	0
17.5-22.5	4	0	4	5	5	0
22.5-27.5	2	0	2	1	1	0
27.5-32.5	4	0	4	5	5	0
32.5-37.5	0	0	0	10	6	4
37.5-42.5	3	0	3	7	3	4
42.5-47.5	0	0	0	1	0	1
47.5-52.5	1	0	1	5	0	5
52.5-57.5	0	0	0	0	0	0
57.5-62.5	2	0	2	3	0	3
over 62.5	1	0	1	1	0	1

compared with that of  $2 \times 10^{-4}$  ergs reported by Coblenz and Fulton (4) as the minimum energy required to kill a bacterium of *B. coli* with ultraviolet light (1700-2700 Å. u). A value somewhat less than  $2 \times 10^{-4}$  ergs may be deduced for staphylococci (5). With electrons, Wells (6) reports the relatively high value of 0.65 ergs as the energy required to kill a single staphylococcus. In these cases, however, the amounts of absorbed radiation remained undetermined. The significance of the calculations is therefore not clear.

Our observation that *Euplotes taylori* is killed less readily than bacteria agrees with the early work of Hertel (7) who studied the lethal

action of ultraviolet light on bacteria, protozoa, coelenterates, annelids, molluscs, amphibia, and plant cells.

Here it should be mentioned that investigations on *Colpidium campylum* carried out by others in this laboratory, suggested that it would be highly desirable to determine whether death by x-rays might not be largely due to the production of toxic products in the medium. With this possibility in mind we irradiated twelve slides of nutrient broth and twelve of yeast autolysate for 9 to 35 minutes ( $2.5 \times 10^3$  Roentgen units per second). After irradiation, six of each received approximately 0.001 cc. of a suspension of *Pseudomonas fluorescens* and the remainder, 0.001 cc. of a suspension of *Bacillus coli*. Pour-plates were made and examined 48 hours later. Growth was equally luxuriant irrespective of whether the plates were prepared from organisms in irradiated or unirradiated media. There was no evidence that irradiation of the medium produced substances of appreciable toxicity to these bacteria.

Eight similar experiments on the effect of irradiated media (1:1 artificial sea water and  $F_1 + K_{13}$ )<sup>5</sup> on *Euplotes taylori* also gave negative results. Although the medium was irradiated for upwards of 30 minutes it failed to be appreciably toxic towards the ciliate. The action of various sensitizers and protective substances on the lethal dose of x-rays for the protozoa and its associated bacteria remains for investigation. It is apparent from theoretical principles and from observations made with x-rays and other sources of radiation (8, 9, 10, 11) that certain changes in the composition of the medium influence materially the lethal dose of radiation. Nevertheless, with *Euplotes taylori* and the bacteria studied here an ordinary medium when irradiated by itself does not become lethal to the organisms.

### PART III

#### *An Attempt to Cultivate Euplotes taylori on Bacteria Killed by X-Rays*

In our earlier work (1) we have shown that *Euplotes taylori* which will thrive on a mixture of two varieties of living bacteria, cannot be

<sup>5</sup>  $F_1$  = *Pseudomonas fluorescens*.

$K_{13}$  = *Bacillus coli*, strain  $K_{13}$ .

maintained upon these same organisms previously killed by heat, toluene, autolysis, phage lysis, or upon sterile dialysates of these bacteria. Since the physical state and chemical composition of bacteria are likely to be the factors which determine their nutritive value for protozoa, it is apparent that the manner of killing is likely to have substantially different effects upon the nutritive value of the bacteria, inasmuch as the end results upon the physical state, enzyme distribution, and chemical composition will be widely different. Recognizing, therefore, that bacteria killed by x-rays would be substantially different from bacteria killed by toluene, heat, lysis, or other means, we were tempted to investigate the possibility of maintaining *Euplotes* upon irradiated bacteria.

In the experiments reported upon in Table III the organisms of Groups 1, 4, and 5 were fed at 3 day intervals upon the same product—equal proportions of  $F_1$  and  $K_{13}$ , suspended in artificial sea water, and subjected to 67 seconds irradiation<sup>6</sup> ( $2.5 \times 10^3$  Roentgen units per second). In Group 4 the irradiated bacteria were also washed twice by centrifuging, the object being to remove water-soluble toxic products produced during irradiation. The protozoa used in these tests were sterilized prior to cultivation in two ways,—by washing (Groups 1 and 4) and by irradiation for 58 seconds (Group 5).

Finally three sets of controls were devised: irradiated protozoa fed upon normal unirradiated  $F_1$  and  $K_{13}$  (Group 7); washed protozoa similarly fed (Group 2); and normal untreated *Euplotes* fed upon normal  $F_1$  and  $K_{13}$  (Group 3). It should be mentioned that the *Euplotes* used in this last control were drawn from a stock laboratory culture which had been maintained for many months in pure line on  $F_1$  and  $K_{13}$ .

The irradiated bacteria and the sterilized protozoa were tested routinely for sterility by the use of heavy inoculations into nutrient broth. All preparations which failed to be sterile were discarded. The table reports only upon cultures which gave satisfactory sterility tests.

In earlier experiments employing very low doses of x-rays (a few seconds exposure) we were interested to notice that the broth media

<sup>6</sup> Note that 15 seconds are sufficient to kill  $F_1$  (*Pseudomonas fluorescens*) and 45 seconds to kill  $K_{13}$ .

used for sterility tests revealed the phenomenon of delayed growth. In a few cases the tubes remained clear for so long as 9 or 10 days (room temperature) and showed such faint cloudiness that we could not be certain for perhaps 48 hours longer whether there was growth. In control experiments on normal unirradiated protozoa the broth used in the sterility tests showed heavy clouding within 24 hours. This phenomenon may be comparable to that displayed by irradiated ciliates (*cf.* Table I) in which sublethal doses of x-rays brought about a marked but temporary cessation of activity.

TABLE III

*Irradiated Bacteria as Food for Euplotes*

Group	No. of cultures tried	Total No. of protozoa used	Initial state of protozoa	Initial state of bacteria ( $F_1 + K_{12}$ )	Life of culture (extremes of entire group)
					<i>days</i>
1	16	$16 \times 6$	Washed	Irradiated	11-23
2	15	$15 \times 6$	Washed	Normal	7 died out within 4 weeks. Remainder in normal condition.
3	22	$15 \times 6$ $7 \times 10$	Normal	Normal	2 cultures died out within 4 weeks. Remainder continued normal
4	16	$16 \times 6$	Washed	Irradiated and washed	13-23
5	30	$30 \times 10$	Irradiated	Irradiated	12-28
6	7	$7 \times 10$	Irradiated	Normal	12-25

Two conclusions follow from the data of Table III. (a) *Euplotes taylori* may not be maintained upon bacteria killed by irradiation (Groups 1 and 5). (b) Even sublethal doses of x-rays cause an insidious damage to the protozoan, as indicated by an ultimate failure in reproduction (Group 7). This injury is not immediately manifest.

## SUMMARY

1. The minimum lethal dose of x-rays for *Euplotes taylori* was determined. Under the conditions of this investigation a 220 second exposure (2110 Roentgen units per second) was required to kill the protozoon. Much less exposure was sufficient to kill the associated

bacteria. This difference in resistance permits the sterilization of protozoa with comparative ease.

2. Irradiation of *Euplotes* for 100 to 220 seconds caused a complete but temporary cessation of ciliary activity in many of the organisms, the percentage so affected increasing with the length of irradiation.

3. Pure cultures of *Pseudomonas fluorescens* and *Bacillus coli*,  $K_{13}$ , separately irradiated, were found to be killed much more readily than protozoa,—the former in 15 seconds exposure (2530 Roentgen units per second) and the latter in 45 seconds.

4. The death of these organisms by irradiation was not due to the action of toxic products in the medium since separately irradiated media were not found to be toxic.

5. Irradiated bacteria were found unsatisfactory for the nutrition of *Euplotes*, previously sterilized either by irradiation or washing.

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*Note Added to Proof.*—Schepman and Flecke (*Klin. Woch.*, 1926, **5**, 1608) irradiated agar-plate cultures of five strains of bacteria with soft x-rays. Although the intensity of the radiation was low (36.1 Roentgen units per second) the lethal dose was of the same order of magnitude as we have reported here. They obtained values ranging between 6500 and 100,000 Roentgen units, which compare favorably with the values of 38,000 and 114,000 observed by us.

# THE VISUAL INTENSITY DISCRIMINATION OF THE HONEY BEE

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## I

The discrimination of luminous intensities has been studied in man in a great many cases (Adams and Cobb, 1922; Aubert, 1865; Blanchard, 1918; Fechner, 1860; Hecht, 1923-24, 1924-25; Koenig, 1895; Koenig and Brodhun, 1889; Lasareff, 1911, 1914; Weber, 1835; Steinheil, 1837). To provide comparable results it has become customary to determine intensity discrimination in such a way that, if  $I$  is the intensity to which the eye is adapted, and  $\Delta I$  is the increase of a second intensity which is supposed to be compared with  $I$  and just so much brighter that it can be distinguished from  $I$ , then the ratio  $\frac{\Delta I}{I}$  is considered a measure of the discriminating power of the eye.

The larger number of studies on intensity discrimination by the human eye was largely due to the fact that one wanted to test the validity of the so called Weber-Fechner law for the eye. According to this rule,  $\frac{\Delta I}{I}$  was supposed to be a constant for any illumination, or at least over a wide range of intensities. This turned out not to be true at all, however; intensity discrimination varies with illumination in a very specific manner.

For vertebrates other than man, and for invertebrates, almost no data are available; at least, no accurate determinations at different intensities have been made which could be compared easily with information on the differential sensitivity of the human eye. In the present experiments intensity discrimination was studied in the compound eye of an arthropod, namely that of the honey bee, which is built and is functionally on a principle entirely different from that of the vertebrate eye.

Bertholf (1931, *a*) has already made a few tests with bees which showed that "the difference between two intensities has to be about 30 per cent if they are to distinguish, whereas the human eye is able to distinguish two intensities when the one intensity is reduced to only 90 per cent of that of the other. Human beings are, therefore, considerably more acute than bees in their ability to distinguish differences in brightness." As there is no possibility of verbal communication between the animal and the experimenter, for such experiments with non-human forms, a conditioned reflex method (Pavlov) or training method (von Frisch) would be generally applied to approach the estimation of intensity discrimination in the bee. Bertholf in his experiments made use of the phototropic response of the bee in such a way that he counted how many times in a certain number of tests a bee would orient to the stronger of two lights.

The methods previously mentioned seemed not reliable enough, however, for obtaining a clear picture of intensity discrimination by the bee, because any conditioning involves a great many steps uncontrollable by the experimenter which might confuse the results. A more direct approach is for this reason highly desirable.

In previous experiments on the visual acuity of the bee (Hecht and Wolf, 1928-29) such a method was successfully used. If the visual field of a photosensitive animal is made up of a pattern of alternating dark and illuminated bars of equal size the animal will respond to any displacement of this field as long as it can distinguish the components of the pattern. In case the animal cannot resolve the pattern the field will appear uniformly illuminated and a displacement will not elicit a response.

For the measurement of intensity discrimination the same method can be applied when instead of the original black and white pattern a pattern is used in which the bars can be changed in brightness. Thus means can be provided to determine at any brightness of one set of stripes the brightness of the other set at which the animal will just show the first response to a motion of the system of stripes.

## II

### *Apparatus and Experimental Procedure*

To obtain a pattern of bars of different brightness a thin opal glass plate was used to which black paper stripes 20 mm. wide were attached, evenly spaced, so

that light shining through the glass produced a pattern of alternating black and white stripes. A width of 20 mm. of the stripes was chosen so that at the lowest illuminations used the visual angle between the edges of one stripe was above threshold angle visual acuity (Hecht and Wolf, 1928-29). When the plate is illuminated at the same time from the other side, the stripes can be made to disappear by changing one or the other light intensity so that the plate looks uniform in brightness.

The actual use of such a test plate for experiments on intensity discrimination can be made out with the help of Fig. 1.

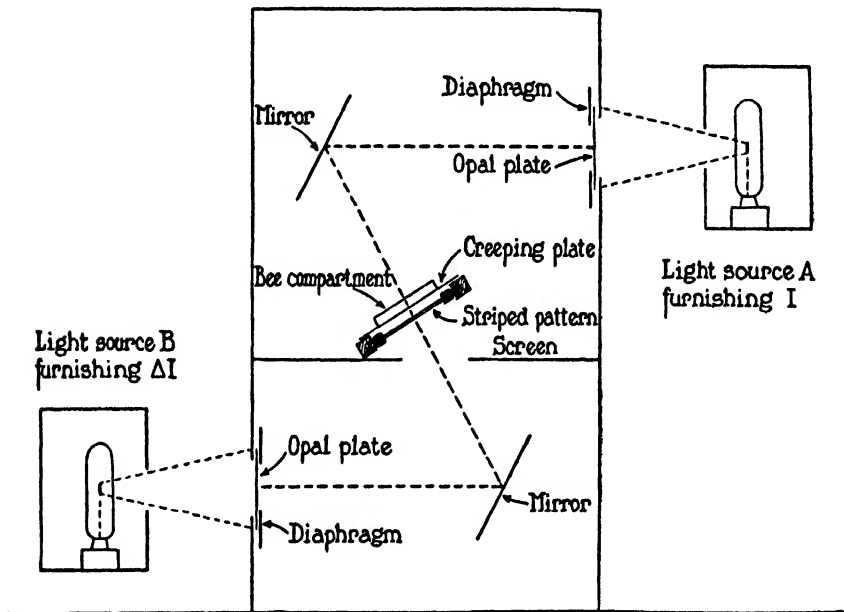


FIG. 1. Diagram of apparatus for measuring the visual intensity discrimination of bees.

Light source *A* (1000 watt, concentrated filament Mazda lamp) illuminates a plate of opal glass in the wall of a dark room in which the experiments are carried on. In the same way (*B*) another source of light (500 watt, concentrated filament Mazda lamp) illuminates an opal plate on the opposite side of the dark room. Directly behind both opal plates are accurately controlled and calibrated diaphragms, the opening of which determines the area of the plates which radiate light into the dark room. The light coming from source *A* is reflected by a mirror and, passing through the bees' creeping cage from above, illuminates the opal plate on top of the striped pattern which makes the whole field look uniformly illuminated. The light coming from source *B* is reflected by a mirror underneath the bees' creeping plane. It passes through the opal plate on all spaces in between the black bars, so that we obtain, seen from above, a pattern of illuminated bars



alternately of different brightness. At all places where black bars are put underneath the opal plate we have the same intensity  $I$ ; at all other places where light from source  $B$  is permitted to pass through we have a higher intensity  $I + \Delta I$ .

The opal plate which bears the black stripes on its lower surface fits into a frame which slides in a pair of grooves in a larger frame and can easily be moved back and forth.

The bee is kept in a glass compartment  $10 \times 10$  cm. and 2 cm. high, which is held about 1 cm. above the pattern. The bottom of the compartment is the plate on which the bee ordinarily crawls. It is an ordinary photographic plate with the halide completely removed but the gelatine left so as to give a foothold for the crawling bee.

The creeping compartment of the bee and the frame with the striped pattern are tilted at an angle of about  $30^\circ$ . The bee, being negatively geotropic and positively phototropic, tends to crawl upward in a straight line so that any deviation from its linear progression due to the movements of the visual field is easily detectable.

The angle at which the bee creeps upward is mostly  $90^\circ$ . According to the knowledge of the geotropic orientation of animals the angle of orientation should be much less than  $90^\circ$  at an inclination of  $30^\circ$  of the creeping plane (Crozier, 1929). In such cases where the purely geotropic orientation of animals is investigated all other factors having any orienting effect, except the gravitational pull, are excluded. In our experiments both gravity and light act upon the animal and the influence of light in a sense predominates over that of gravity and causes the bee to crawl up straight.

The illumination of the striped visual field of the bee can be varied in two ways. Both light sources are mounted on optical rails and can be moved closer or further away from the opal plates. To calibrate the apparatus accurately for source  $A$  only three fixed positions were chosen: Position I, 15 cm. from the opal plate in the wall of the dark room; Position II, 91 cm.; Position III, 182 cm. from the opal plate. For source  $B$  the distances from the opal plate were: Position I, 40 cm.; Position II, 150 cm. To both diaphragms engraved metal scales were attached to one of the two leaves, which could be read to  $\frac{1}{16}$  of a millimeter. For eleven different settings of the diaphragms furnishing  $I$  and  $\Delta I$  the actual brightness was measured by means of a Leeds and Northrup Macbeth illuminometer. The measured points, plotted as scale reading on the diaphragm against the logarithm of the resulting brightness, fall on smooth curves for each position of the light sources. These, drawn on a sufficiently large scale, were used as calibration curves.

The experiments are carried on as follows. Worker bees are caught in front of the hive and brought into the laboratory. To clip their wings they are lightly anesthetized. They recover after about  $\frac{1}{2}$  hour and are kept in wire cages where they have access to honey. A special feeding device is built into every cage which gives them plenty of food without getting them sticky, as this might handicap the bees during the tests.

The response to the moving stripes is such that among a total of 50 to 60 bees used in 1 day only very few do not react at the time of the test, so that practically any bee can be used for observation.

To make a measurement, the lights from both sources are turned on, and the diaphragm in front of source *A* is set for a definite illumination. The diaphragm in front of source *B* is wide open, so that the pattern looks almost as if composed of black and white stripes. A bee is put into the compartment. Generally the animal is very active, and for the most part it creeps upward on the lower side of the compartment, then continues its journey downward on the cover of the compartment and again begins its journey in the same manner as before.

In the visual acuity tests carried on some years ago (Hecht and Wolf, 1928-29), the bees stayed most of the time on the bottom plate; in these experiments where we have at the same time an illumination from above, the tendency to creep dorsum down on the under side of the cover of the compartment is very much greater. There is however always a great probability that the bees will creep on the bottom, so that tests can be made easily. While the bee is moving on the bottom and the direction of progression is clear, the pattern plate is moved with a sharp but not too rapid motion. Depending upon whether the bee responds or not, the intensity of the light coming from underneath the pattern plate is lowered or raised to make the contrast between the stripes sharper or weaker, until an illumination is reached at which the bee just responds to the movement.

Under these conditions, the bee changes its direction of creeping quite precisely. If the pattern is moved in one direction the bee swings over in the opposite direction—moving against the motion of the stripes—and continues creeping in the new direction. For a great many cases, provided that the visual angle of the stripes is not below threshold angle for a given illumination, the response occurs even in bees creeping on the top side of the compartment, dorsum down.

The body of the bee throws a shadow on the creeping plane with the stronger light above, which might interfere with the bee's response. According to the anatomical findings on the bee's eye (Baumgärtner, 1928) the "fovea" which is probably mostly concerned with the dissolution of details occupies a lateral portion of the eye and is directed laterally so that the shadows cast by the bee's body can hardly interfere with the field of vision of either eye.

The response to the moving pattern is generally not quite as precise however as it was in the tests of visual acuity, where we had to deal with alternating black and white bars. The results obtained, however, give a clear picture of the intensity discrimination of the bee's eye as a function of illumination.

During the experiments about ten bees were tested at a given intensity provided from source *A* for which the necessary intensities of *B* were determined. Then the diaphragm in front of *A* was set for another intensity and another ten bees tested and so forth until the whole range desired was covered. Knowing that there are not many

individual differences among bees of the same colony most of the data were obtained in such a way that only one single determination was made with each bee used.

For another set of experiments some marked bees were used for testing the whole or at least a greater part of the range of intensities. Such tests showed that there is only a negligible difference in the results obtained by the two methods. A single individual, however, never gives as good a complete curve as a larger group, which is probably due to the handling of the animals so many more times.

### III

#### RESULTS

Using the experience collected during the work on the visual acuity of the bee the study on intensity discrimination could be carried on within a short time after the method of testing bees was developed and the apparatus built.

Table I gives the values for intensity discrimination over a range of intensities from about 1/100 to 100 millilamberts. This range corresponds to the one used for the study of visual acuity. Below 1/100 millilambert the visual acuity of the bee is so poor that there was no need to make a test at lower intensities. In the same way, at high intensities the upper limit was reached above which any increase in brightness no longer improves the resolving power of the bee's eye.

Over the desirable intensity range 270 bees were tested at twenty-seven different intensities within that range. Generally ten bees were tested at each intensity, in some cases a few more, and in one case less than ten. The values of  $I$  and of  $\frac{\Delta I}{I}$  given in Table I are *mean* values for the total number of individuals tested at each  $I$ , with the probable errors of  $\frac{\Delta I}{I}$  and  $\Delta I$  computed according to Peter's formula.

The values for  $\frac{\Delta I}{I}$  given in Table I vary in a significant manner with illumination. At low intensities  $\frac{\Delta I}{I}$  is greatest; it decreases smoothly as the illumination is increased. The probable error of the mean  $\Delta I/I$  changes in a similar way, decreasing with increasing brightness.

In Fig. 2 the data are presented graphically. The points plotted are the mean values for all individuals tested at the respective intensities.

TABLE I

*Mean Values for Intensity Discrimination at Different Intensities Measured in Millilamberts with Their Probable Errors ( $n = 10$ )*

No. of individuals $n$	$I$	$\log I$	$\Delta I$	$\frac{\Delta I}{I}$	P. E. $m \frac{\Delta I}{I}$	P. E. $m \Delta I$
10	0.0417	$\bar{2}.620$	0.2270	5.5454	$\pm 0.2878$	$\pm 0.0122$
10	0.0564	$\bar{2}.751$	0.1841	3.2584	0.1941	0.0104
10	0.0628	$\bar{2}.798$	0.1478	2.3536	0.1761	0.0110
10	0.0759	$\bar{2}.880$	0.1644	2.1671	0.0801	0.0062
12	0.1072	$\bar{1}.030$	0.2104	1.8790	0.0802	0.0078
10	0.1337	$\bar{1}.126$	0.2187	1.6518	0.0787	0.0094
10	0.1664	$\bar{1}.221$	0.2166	1.4021	0.0673	0.0104
10	0.2138	$\bar{1}.330$	0.2120	0.9915	0.0467	0.0106
10	0.3020	$\bar{1}.480$	0.2587	0.8568	0.0345	0.0104
10	0.4169	$\bar{1}.620$	0.3482	0.8350	0.0463	0.0193
10	0.5496	$\bar{1}.740$	0.3442	0.6262	0.0272	0.0149
10	0.6951	$\bar{1}.842$	0.4790	0.6893	0.0338	0.0233
10	1.031	0.013	0.6324	0.5704	0.0333	0.0445
10	1.439	0.158	0.6381	0.4435	0.0222	0.0354
10	2.153	0.333	0.5695	0.2645	0.0158	0.0342
10	2.711	0.433	0.8842	0.3261	0.0096	0.0260
5	3.900	0.591	0.9308	0.2387	0.0096	0.0374
12	5.261	0.721	1.6116	0.3064	0.0164	0.0859
10	6.792	0.832	1.9706	0.2902	0.0065	0.0571
10	9.572	0.981	3.1607	0.3302	0.0200	0.1634
10	13.87	1.142	3.4357	0.2482	0.0168	0.2318
10	19.96	1.300	5.6661	0.2838	0.0151	0.2908
10	27.42	1.438	5.6387	0.2056	0.0054	0.1471
10	34.68	1.540	9.3068	0.2684	0.0094	0.3246
11	54.75	1.739	14.187	0.2595	0.0066	0.3538
10	82.01	1.914	21.599	0.2633	0.0051	0.4159
10	108.39	2.035	24.458	0.2258	0.0057	0.6458

The graph shows that the relation between intensity discrimination and the logarithm of the illumination is of pretty much the same character as with the human eye. At low illumination the discrimination is poor, and as illumination increases it becomes better, up to a certain maximum beyond which the intensity discrimination cannot

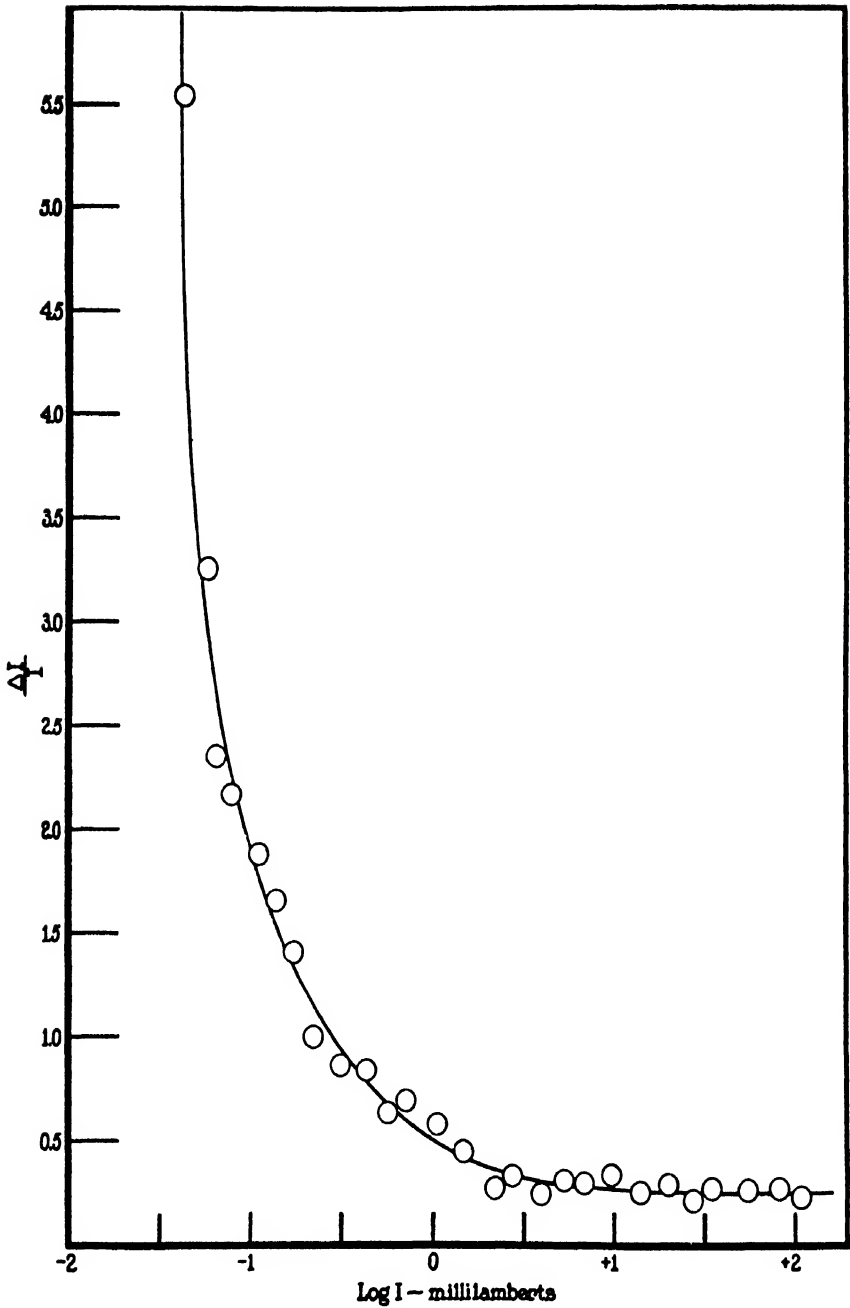


FIG. 2. Relation between intensity discrimination and illumination. Single readings from 270 bees at twenty-seven different intensities. The points represent averages of the total number of bees tested at each illumination.

be further improved. The intensity range and the absolute values for discrimination at each intensity are, however very different from the figures for the discriminating power of the human eye.

TABLE II

*Values for Intensity Discrimination of Numbered Animals Tested Repeatedly at Different Intensities*

Individual No.	Log I								
	2.759	2.880	1.330	1.620	1.842	0.333	0.591	1.300	1.540
1		2.582	1.490		0.826	0.263		0.189	
2		2.878	1.183		0.601	0.233		0.186	
3		2.489	1.472		0.725	0.214		0.291	
4		2.191	1.097		0.617	0.352		0.200	
5		2.903	1.228		0.692	0.269		0.211	
6		2.488	1.021		0.692	0.299		0.307	
7		2.095	1.183		0.793	0.296		0.223	
8		2.439	0.957		0.599	0.210		0.233	
9		2.755	0.811		0.826	0.356		0.224	
10		1.578	1.074		0.617	0.355		0.200	
11	3.069			0.792			0.234		—
12	3.928			0.986			0.214		0.295
13	4.488			1.271			0.229		0.344
14	—			0.912			0.227		0.251
15	2.449			0.726			0.268		0.317
16	2.945			1.122			0.214		0.226
17	3.708			0.929			0.232		0.263
18	3.326			0.957			0.198		0.251
19	4.247			0.897			0.217		0.262
20	2.748			0.815			0.266		0.227
21	2.918						0.291		0.280
	3.383	2.440	1.149	0.941	0.699	0.285	0.236	0.226	0.272

To investigate whether there is any difference between the intensity discrimination curve given in Fig. 2 where only one single test was made with each individual bee used, in another set of experiments bees were marked with paint spots on the thorax and abdomen and their discriminating power was tested several times at different illumi-

nations. These data are given in Table II. The values on the first line are the logarithms of the intensities at which the tests were made. The vertical columns give the corresponding values of  $\frac{\Delta I}{I}$  for each individual.

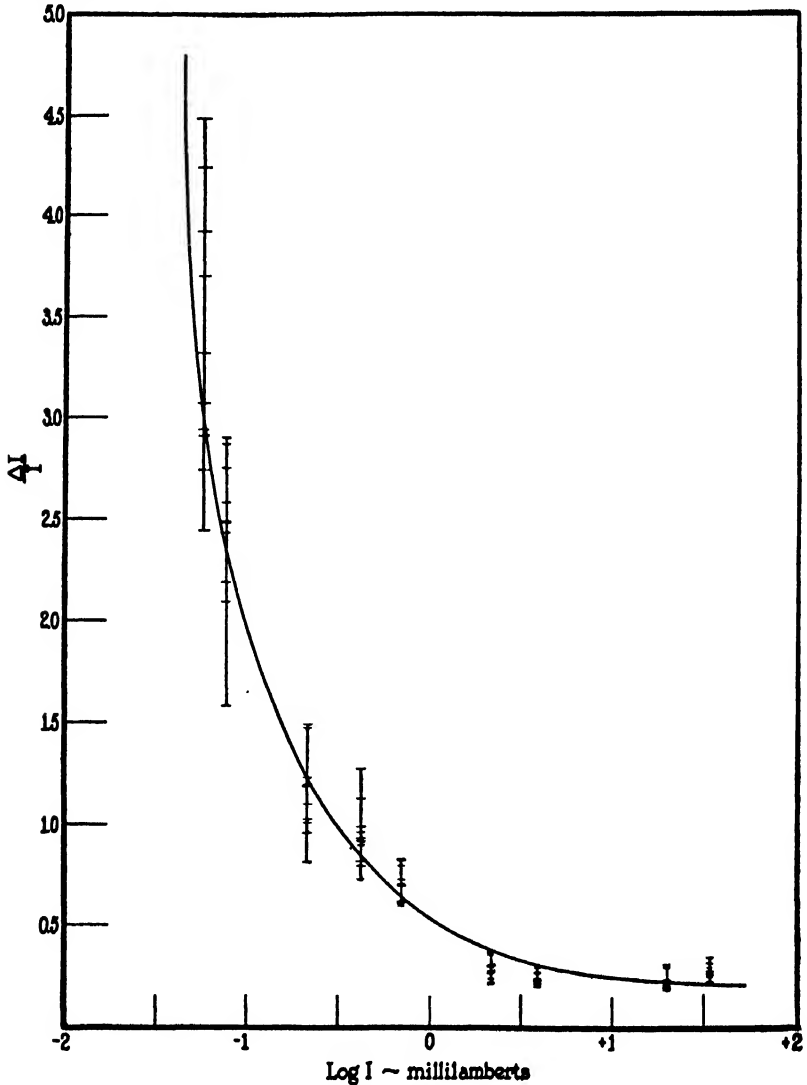


FIG. 3. Relation between intensity discrimination and illumination data from twenty-one individuals tested repeatedly at different intensities. Each horizontal bar corresponds to one observation. The curve is the same as in Fig. 2 to show whether there is a difference between single or repeated tests.

In Fig. 3 the data of Table II are given graphically. On account of the fact that at high intensities the points fall very close together, the readings taken with each individual are not presented with different symbols. Each horizontal bar through the vertical lines represents one point. The curve drawn through the points is the same as that given in Fig. 2. This has been done to show whether there is a significant difference according to the way in which the data have been collected. The fit of the curve is not perfect, but at least it is adequate enough to show that in repeated tests of the discriminating power of the eye of a single individual the same data are obtained as in single tests with different bees.

## IV

The variations of the determinations of  $\Delta I$  are of some special interest. An actual measurement is made by setting the intensity ( $I + \Delta I$ ). Two influences can affect such a setting: (1) inadequacy due to lack of "fineness" or "nicety" of the adjustment by the observer; (2) the natural, inherent, variation of sensitivity in a bee, from moment to moment, and among the population of bees. It may be important to discriminate between these two kinds of variation, and if possible to obtain an indication of the inherent self-consistency of the data. For analysis of such relationships it is essential that the means be based upon a constant number of observations throughout (*cf.* Crozier, 1929, Crozier and Pincus, 1931-32; Hoagland and Crozier, 1931-32). It would be expected that with decrease of  $\Delta I$  for a just detectable effect, which of course depends upon lower  $I$ , *sharper* settings of ( $I + \Delta I$ ) should be possible as concerns absolute error of adjustment; at the same time fluctuations in relative sensitivity of the bees should be of absolutely greater effect upon the measured  $\Delta I$  over a very wide range of values of  $I$ ; we would then look to have  $P.E._{\Delta I}$  pass through a minimum. We find indeed, that  $P.E._{(\Delta I/I)}$  decreases with increasing  $I$  exactly in the same way as does  $\frac{\Delta I}{I}$  itself (Fig. 4), and that mean

$\Delta I$  and  $P.E._{\Delta I}$  increase together (Table I). The dependence of  $P.E._{\Delta I}$  upon  $I$  is exhibited in Fig. 5.  $\log P.E._{\Delta I}$  is, with sufficient precision, a rectilinear function of  $\log I$ . The determinations at the very lowest values of  $I$  are suggestive of the minimum in the curve of  $P.E._{\Delta I}$



to which we have referred; the experience with the measurements very definitely suggests that with still lower intensities the standard deviation of  $\Delta I$  increases enormously. In the range of higher intensities,

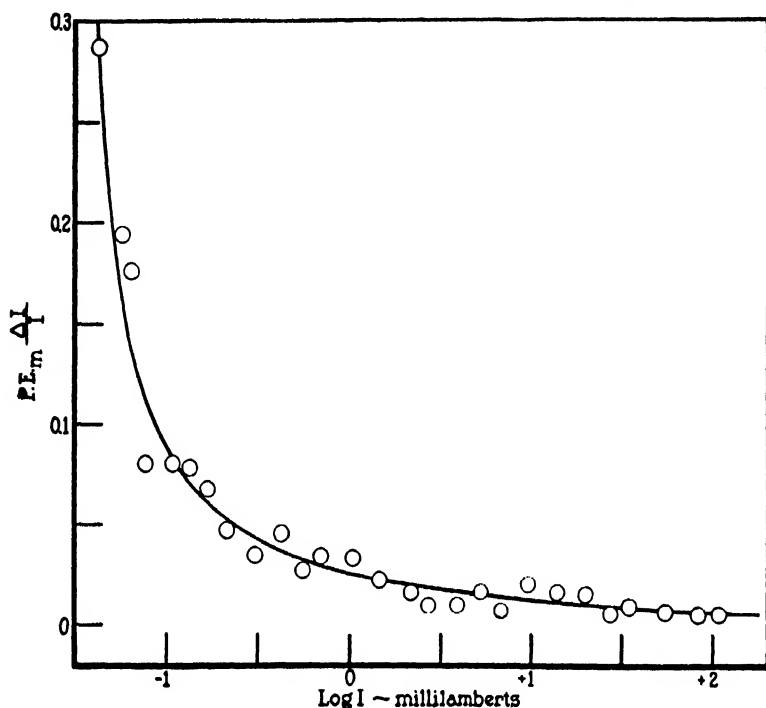


FIG. 4. Relation between the probable error of  $\frac{\Delta I}{I}$  and illumination.

P. E.  $(\Delta I/I)$  decreases with increasing light intensity in the same way as  $\frac{\Delta I}{I}$ .

however, where satisfactorily reproducible settings are obtainable,  $\log \text{P.E.}_{\Delta I}$  is directly proportional to  $\log I$ . This means that as  $I$  increases the relative change of  $\text{P.E.}_{\Delta I}$  (i.e.  $\frac{\Delta \text{P.E.}_{\Delta I}}{\text{P.E.}_{\Delta I}}$ ) is directly proportional to  $\frac{\Delta I}{I}$ . Hence we are at liberty to conclude (1) that the

measurements exhibit an internal self-consistency which is beyond accident, and (2) that the excitation factors which determine  $\Delta I$  also determine and control the *variation* of  $\Delta I$ ; hence that the quantitative level of intensity discrimination determines, or limits, the variation in the measure of this discrimination.

## V

Having provided an intensity discrimination curve for the bee's eye over a range of intensities of three and a half logarithmic units, it is of some interest to compare the efficiency of the bee's eye in this respect with the human eye.

Hecht (1924-25) has computed from the data of earlier investigators an intensity discrimination curve for the human eye. From his

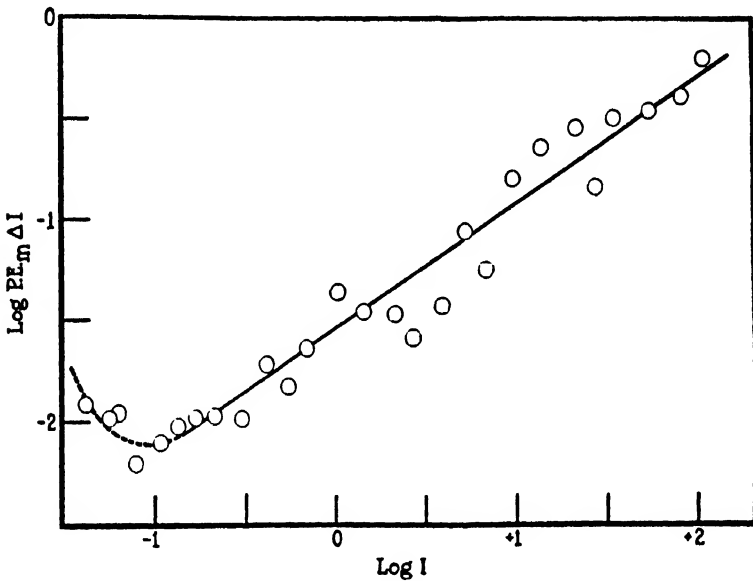


FIG. 5. Relation between the logarithm of the probable error of  $\Delta I$  and illumination.  $\text{Log P.E. } \Delta I$  is a rectilinear function of  $\text{log } I$  above a certain value for  $I$ . At very low intensities  $\text{P.E. } \Delta I$  increases.

curve values can be obtained that can easily be compared with those now established for the bee. Table III shows that the human eye is able to distinguish between two lights of different brightness at very much lower illuminations than can the bee's eye. At light intensities below 1/100 millilambert the visual acuity of the bee's eye is so poor that it cannot perceive at all the stripe system in front of it (Hecht and Wolf, 1928-29). At an illumination of 1/10 millilambert the bee's intensity discrimination is about 2 units, which is about three times as bad as it is for the human eye at its worst. The human eye has at that illumination a discrimination power which is thirty times

better than in the bee; and at an illumination of 100 millilamberts, where for both the human eye and the bee's eye the intensity discrimination has reached the maximum, the bee's eye is capable only of an intensity discrimination which is twenty times worse than for the human eye.

TABLE III

*Comparison of the Intensity Discrimination of the Human Eye and the Bee's Eye at Different Illuminations*

Log I millilamberts	Human eye	Bee's eye
-5.5	0.77	—
-4.0	0.52	—
-4.5	0.35	—
-3.0	0.23	—
-3.5	0.14	—
-2.0	0.085	—
-2.5	0.05	—
-1.0	0.04	1.93
-1.5	0.03	0.95
0.0	0.02	0.51
+0.5	0.017	0.33
+1.0	0.015	0.26
+1.5	0.015	0.25
+2.0	0.015	0.24
+2.5	0.018	—
+3.0	0.02	—
+3.5	0.03	—

## SUMMARY

1. Bees respond by a characteristic reflex to a movement in their visual field. By confining the field to a series of parallel stripes of different brightness it is possible to determine at any brightness of one of the two stripe systems the brightness of the second at which the bee will first respond to a displacement of the field. Thus intensity discrimination can be determined.

2. The discriminating power of the bee's eye varies with illumination in much the same way that it does for the human eye. The discrimination is poor at low illumination; as the intensity of illumination increases the discrimination increases and seems to reach a constant level at high illuminations.

3. The probable error of  $\frac{\Delta I}{I}$  decreases with increasing  $I$  exactly in the same way as does  $\frac{\Delta I}{I}$  itself. The logarithm of the probable error of  $\Delta I$  is a rectilinear function of  $\log I$  for all but the very lowest intensities. Such relationships show that the measurements exhibit an internal self-consistency which is beyond accident.

4. A comparison of the efficiency of the bee's eye with that of the human eye shows that the range over which the human eye can perceive and discriminate different brightnesses is very much greater than for the bee's eye. When the discrimination power of the human eye has reached almost a constant maximal level the bee's discrimination is still very poor, and at an illumination where as well the discrimination power of the human eye and the bee's eye are at their best, the intensity discrimination of the bee is twenty times worse than in the human eye.

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# THE DISTRIBUTION OF THE ACTION CURRENTS PRODUCED BY HEART MUSCLE AND OTHER EXCITABLE TISSUES IMMERSSED IN EXTENSIVE CONDUCTING MEDIA\*

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## INTRODUCTION

In their natural environment the excitable tissues of living organisms are surrounded by a more or less extensive medium which contains electrolytes. It is necessary, therefore, in any investigation of the electrical activities exhibited by such tissues *in situ* to take into consideration the character of the laws that govern the distribution of electric currents in volume conductors.

The writings of Waller (1889) and of Einthoven and his associates (1913) indicate that these investigators were familiar with these laws and appreciated their importance. Wilson, Wishart, and Herrmann (1926) pointed out that they determine the distribution of the electric currents produced by the heart and have an important bearing upon many electrocardiographic problems. A more complete account of the observations upon which this preliminary report was based has recently been published (Wilson, 1930). Craib (1927, 1928, 1930) has shown that the electric field produced by the excitation or injury of strips of cardiac muscle, skeletal muscle, or medullated nerve immersed in an extensive conducting medium is similar to that which surrounds an electrical doublet. His observations led him to advance the view that at the moment of excitation doublets develop at the surface of or at phase boundaries within the excitable tissue. Bishop

\* A preliminary report based on the material incorporated in this article appeared in *Proc. Soc. Exp. Biol. and Med.*, 1930, **27**, 588.

and Gilson (1929) have confirmed some of the observations made by Craib on skeletal muscle, but interpreted them in a different manner.

It is our purpose to apply the laws that govern the flow of electric currents in volume conductors to the analysis of certain curves obtained by leading directly from the surface of the mammalian auricle<sup>1</sup> and to explain our observations and those made by investigators working with other tissues in terms of the membrane theory.

### *Distribution of Electric Currents in Volume Conductors*

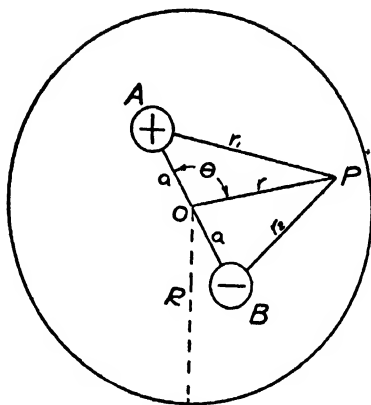
The exact form of the equation that defines the distribution of an electric current flowing in a volume conductor depends upon many factors. The equations that apply in several cases of a simple kind

<sup>1</sup> The mammalian auricle is not essentially a more complicated structure than a curarized frog's sartorius, an isolated nerve, or a strip of cardiac muscle cut from the turtle's ventricle. It can be studied *in situ* in the uninjured state; and, unlike a nerve or a skeletal muscle, which is made up of individual fibers differing one from another in their physiological properties, it responds as a single unit. The cardiac impulse arises spontaneously at a known site, and spreads over the auricular syncytium in essentially the same way as it might spread from a point of origin over a simple protoplasmic sheet.

It may be pointed out, however, that the work of Craib (1927, 1928, 1930) shows that there is no essential difference between the distribution of the currents of action and of injury produced by strips of cardiac muscle, skeletal muscle, or nerve immersed in an extensive conducting medium and those produced by heart muscle under the conditions of our experiments. Between Craib and ourselves there is complete agreement as far as the experimental facts are concerned; in the majority of instances there is, furthermore, no important difference in opinion between us as to the interpretation that should be placed upon these facts.

In stating that work on the general subject of this article was begun in this laboratory a number of years before Craib's first paper was published (see Wilson, Wishart, and Herrmann, 1926, and Wilson, 1930) and that many of the facts to which he has called attention were known to one of us long before that paper appeared, we do not wish to raise any question of priority or in any way to claim any share of the credit due Craib for the fine work he has done. Inasmuch as our work has been done independently of his, we have naturally followed our own point of view. Craib's work has, however, made it unnecessary for us to carry out experiments upon simple tissue strips or to enter at length upon subjects that have been adequately discussed in his papers.

The interpretation which Bishop and Gilson (1929) have placed upon experimental results similar to those obtained by Craib is, in our opinion, in conflict with the laws that define the flow of electric currents in volume conductors.



$$V = c \log_e \frac{r_2}{r_1} \quad (1)$$

$$V = 2ac \cos \theta \quad (2)$$

$$V = 2ac \cos \theta \left( \frac{1}{r} + \frac{r}{R^2} \right) \quad (3)$$

$$V = c' \left( \frac{1}{r_1} - \frac{1}{r_2} \right) \quad (4)$$

$$V = 2ac' \cos \theta \quad (5)$$

$$V = 2ac' \cos \theta \left( \frac{1}{r} + \frac{2r}{R^2} \right) \quad (6)$$

FIG. 1.  $Q$  units of positive electricity per second enter an extensive homogeneous medium through a surface of small radius at  $A$  and the same quantity leaves the conductor per second through a similar surface at  $B$ .  $V$  is the potential of any point  $P$  of the conductor. Equations (1) and (2) apply to an infinite plane lamina; equations (4) and (5) to a medium that is infinite in all directions. Equation (3) applies to a circular lamina of radius  $R$  and equation (6) to a sphere of radius  $R$ . In equations (2) and (5) it is assumed that  $a$ , one-half the distance between the source and sink, is very small in comparison with  $r$ . Equations (3) and (6) involve the additional assumption that the sink and source are equidistant from  $O$ , the center of the circular lamina or sphere.  $c$  equals  $\frac{Q}{2\pi kd}$  and  $c'$  equals  $\frac{Q}{4\pi k}$ , where  $d$  is the thickness of the lamina and  $k$  the specific conductivity of the material of which the conductor is composed. The line  $AB$  may be called the axis of the potential difference, or of the field. For the derivation of the equations, see Pierce (1902), Mason and Weaver (1929), Canfield (1927), Jeans (1923), and Wilson (1930).



are given in Fig. 1; for our present purpose there is no advantage in dealing with more complicated conditions. Examination of these equations shows that the potential is positive at points that are nearer the source than the sink and negative at points that are closer to the latter than the former. It is zero at points equidistant from the source and sink and approaches zero at points that are at a great distance from either.

When  $r$  is very small in comparison with  $R$ , equations (5) and (6) become identical. Consequently, when the potential of a point that is very near to a source and sink that lie close together in an extensive medium is under investigation, the conductor may be considered infinite for all practical purposes. When  $r$  is equal to  $R$  the expression  $\left(\frac{1}{r^2} + \frac{2r}{R^3}\right)$  in equation (6) becomes  $3/r^2$ ; *i.e.*, at any point upon the surface of the sphere the magnitude of the potential is three times as great as it would be at the same point if the medium were infinite.

It may also be pointed out that if the volume conductor is infinite in all directions one of the two parts into which it is divided by passing a plane through the source and sink may be removed without altering the character of the electric field<sup>2</sup> (Pierce, 1902, p. 243).

The application of the equations of Fig. 1 to the analysis of curves obtained from the heart *in situ* involves some rather sweeping assumptions. The equations apply to steady currents, while the currents produced by the heart are constantly varying. It is assumed, therefore, that the effects of induction and capacity are not important. The conducting medium in which the heart is immersed is not homogeneous, as the equations require, but heterogeneous. Experience has shown, however, that the currents produced by the heart are distributed in the same general way as they would be if the heart were surrounded by an extensive homogeneous conductor. Einthoven's equilateral triangle is based on the assumption that this is the case,

<sup>2</sup> The electric currents that arise in the muscle fibers at the surface of the auricle will therefore be distributed in much the same way after the auricle is exposed by opening the chest as before. When the sink and source do not lie in the plane surface of the conducting half-space obtained by passing a plane through the infinite medium but beneath it the equation that defines the potential of points lying in this surface is of the same form as if the space were whole.

and has nevertheless been found satisfactory for all practical purposes. Nernst (1908) has shown that in such conductors as salt solutions and organic tissues the distribution of steady and of alternating currents is not materially different.

### *Experimental Curves*

With these considerations in mind we may now turn to an experiment which, so it seems to us, is of a fundamental kind.

The curves shown in Fig. 2 A were obtained in the following way. A large dog was anesthetized with sodium amytal, the chest was opened, and the heart exposed. A non-polarizable electrode of small diameter was placed upon the surface of the right auricle midway between the upper end of the sulcus terminalis and the tip of the right auricular appendix. This electrode was attached to the right-arm terminal of the string galvanometer. The other terminal was attached to a similar electrode in contact with the subcutaneous tissues of the left hind leg.<sup>3</sup> The galvanometer was used at one-fifth the normal sensitivity. Two other electrodes of the same kind, the first in contact with a point near the junction of the superior vena cava and right auricle and the second upon the right hind leg, were attached to the terminals of a second galvanometer so arranged that the deflections of both instruments might be recorded on the same plate. The second galvanometer was used merely for the purpose of obtaining a standard curve to which the events recorded by the first galvanometer might be related.

It will be observed that the auricular curve recorded by the first galvanometer, (upper curve of Fig. 2 A), aside from rather complicated minor details, is of relatively simple form. The gradual descent, indicating relative positivity of the exploring electrode, with which it begins, becomes steeper and steeper until a sharp inverted peak is reached. Then there is a very sudden shift to a peak similar in all respects to the first, except that it is above the base line. The succeeding descent of the curve begins with a steep slope which becomes more and more gradual until the zero position is attained.<sup>4</sup>

<sup>3</sup> Hereafter we shall refer to the electrode placed on or near the heart as the exploring, proximal, or cardiac electrode; and to the electrode placed at a distance from it as the indifferent, distal, or leg electrode. In these leads the galvanometer attachments are made in such a way that relative negativity of the exploring electrode yields an upward deflection in the completed record.

<sup>4</sup> These deflections correspond to the *P*-deflection of the ordinary electrocardiogram, and have approximately the same total duration if we measure from the

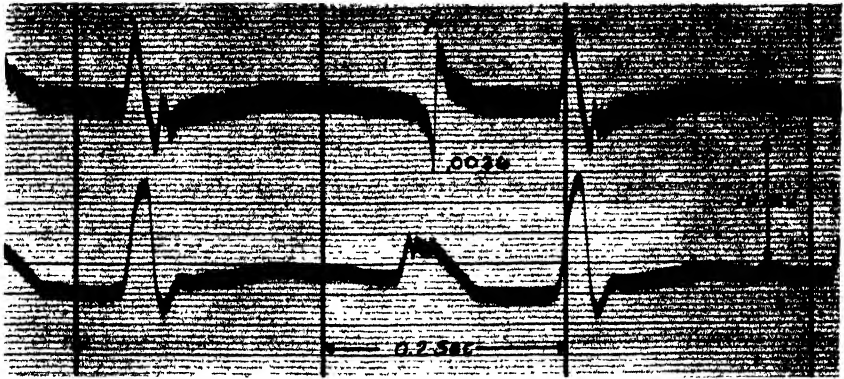


FIG. 2 A. *Upper curve*: Direct lead from a point on the surface of the right auricle of a large dog, midway between the upper end of the sulcus terminalis and the tip of the auricular appendix. An upward deflection indicates relative negativity of the exploring electrode. Indifferent electrode on left hind leg. 10 mv. equals 2 cm. Time marks indicate fifths of a second. Deflection time of the string for 10 mv. about 0.0028 second. The string was not quite aperiodic and slight overshooting was present. The "intrinsic deflection" (upward stroke) has a duration of about 0.0036 (Lucas comparator) and occurs 0.0309 after the first auricular deflection of the standard curve (lower curve).

*Lower curve*. Exploring electrode near junction of superior vena cava and right auricle, possibly on the vein. Indifferent electrode on right hind leg.

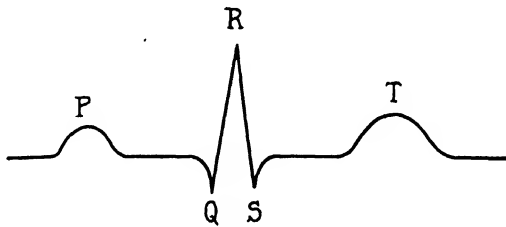


FIG. 2 B. Normal electrocardiogram

first departure from the base line to the final return. They are the result of the spread of the excitatory process over the auricular muscle. In another sense they correspond to the initial deflections of the ventricular complex as opposed to the final deflection, for they are followed by a slow deflection of the same type as the T-wave. This deflection is hidden in the particular curve under consideration by the occurrence of large deflections of ventricular origin, which occur in their proper relation to auricular systole, but is clearly seen in curves from animals in which heart-block was produced.

A curve of the same type as that described was obtained in an experiment in which, after clamping and cutting off the tip of the auricular appendix, the exploring electrode was introduced into the auricle and brought into contact with the endocardial surface of the auricular wall in the same region as in the external leads. This curve was very slightly reduced in amplitude when a thick pad of gauze soaked in Ringer's solution was placed in contact with the external surface of the auricle, and regained its original amplitude when this pad was removed.

From what has been said previously it is clear that the distal electrode placed upon the left hind leg was so far from the auricle, where all of the currents in which we are at present interested were produced, in comparison with the cardiac electrode, that its potential variations may be regarded as negligible.<sup>5</sup> We may therefore regard the upper curve of Fig. 2 A as a record of the potential variations of the small region of the auricular surface upon which the proximal electrode was placed. The spread of the excitatory process from the sinus node over the auricular muscle produced at this point, if we may refer to it by that name, a gradual increase in positivity to an abrupt maximum, a sudden reversal to maximum negativity, and a final decline to zero.<sup>6</sup> When curves taken at points nearer to and at points farther from the sinus node are examined it is found that the peak of maximum positivity and the succeeding peak of maximum negativity retain the same, or nearly the same, relation to each other, but alter their relation to the standard curve, coming at a progressively later time as the distance of the point investigated from the sinus node increases (Fig. 9). Obviously, the peak of maximum positivity

<sup>5</sup> The elimination of the influence exerted by variations in the potential of the leg electrode upon the curve under consideration could not possibly alter any ordinate of this curve by more than two or three-tenths of a millivolt or approximately one-half of one small scale division (*cf.* Wilson, F. N., Macleod, A. G., and Barker, P. S., *Am. Heart J.*, 1931, 7, 207).

<sup>6</sup> This curve may be compared with those that Craib obtained by a similar method of leading from strips of cardiac muscle, skeletal muscle, and nerve immersed in an extensive conducting medium (1930, Plate I, Curves 5 to 10 inclusive). In making this comparison it should be borne in mind that the deflections that Craib ascribes to "doublets of retreat" are hidden in Fig. 2 A by the occurrence of ventricular deflections.

marks the onset of what Lewis and his associates (1914) have referred to as the "intrinsic deflection." As they have shown, this deflection signals the arrival of the excitation process beneath the proximal contact of a pair arranged radially with reference to the sinus node.<sup>7</sup>

It will be noted, however, that the potential of the exploring electrode does not actually become negative until the quick upstroke that follows the inverted peak crosses the base line. It will be convenient to regard this latter point as marking the passage of the crest of the excitation wave.

In attempting to analyze the curve under consideration we may assume, for the moment, that we are dealing with a single source and a single sink. While the first half of this curve was being written, the exploring electrode was positive and therefore nearer to the source than to the sink; during the period when the last half was written it was negative and therefore nearer to the latter than to the former. The form of the curve clearly suggests that the crest of the excitation wave is preceded by a source and followed by a sink.

### *Theoretical Curves*

With the help of this suggestion we may attempt to derive an equation that will define a curve similar to that recorded experimentally. For the purpose of investigating the effect of a single source and a single sink moving in a straight line with a uniform velocity upon the potential of a point near which they pass, we may utilize equation (4).

The axis of the potential difference may be taken as the axis of  $X$ , and the point midway between the source and sink as origin. The potential of  $P$ , any point of the line  $y = b$ , may then be determined in the following way: in Fig. 3,  $r_2$ , the distance of  $P$  from the source, equals  $\sqrt{(x - a)^2 + b^2}$ , and  $r_1$ , the distance of the same point from the sink equals  $\sqrt{(x + a)^2 + b^2}$ . Consequently, by substitution in equation (4) we have

<sup>7</sup> In the article referred to the electrode that was nearer the sinus node was called the proximal electrode; it was attached to the right-arm terminal of the string galvanometer.

$$V = c' \left( \frac{1}{\sqrt{(x-a)^2 + b^2}} - \frac{1}{\sqrt{(x+a)^2 + b^2}} \right) \quad (7)$$

The quantity  $b$  is obviously the value of  $r_2$  when  $x = a$  and the value of  $r_1$  when  $x = -a$ . It represents the minimal distance between the point  $P$  and the sink or source. It is necessary to introduce this quantity in order to avoid infinite values of  $V$  when  $r_1$  or  $r_2$  becomes zero, and which result from the assumption that the source and sink may each be represented by a point.

The form of the curve represented by equation (7) for arbitrary values of  $c'$ ,  $a$ , and  $b$  is shown in Fig. 4. It will be observed that this curve closely resembles the experimental curve described insofar as the general outline of the latter is concerned. It is obvious that when

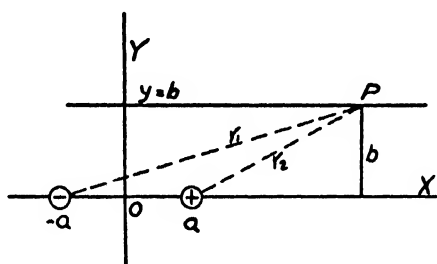


FIG. 3

$x = 0$ ,  $V = 0$ , and that when  $b$  is small,  $V$  is maximal for some value of  $x$  slightly greater than  $a$  and minimal for some value of  $x$  slightly less than  $-a$ . We have not been able to determine the maxima and minima of this curve analytically, but this can be done in the case of the corresponding curve derived from equation (5). In that case

$$V = 2ac' \frac{x}{(x^2 + b^2)^{3/2}} \quad (8)$$

and  $V$  is maximal when  $x = b/\sqrt{2}$  and minimal when  $x = -b/\sqrt{2}$ .

In the curves represented by both of these equations (7 and 8) the maximal and minimal values of the function depend upon  $b$  in such a way that the distance between the positive and negative peaks increases as  $b$  increases. In equation (7) this distance approaches the limit  $2a$  as  $b$  approaches zero.

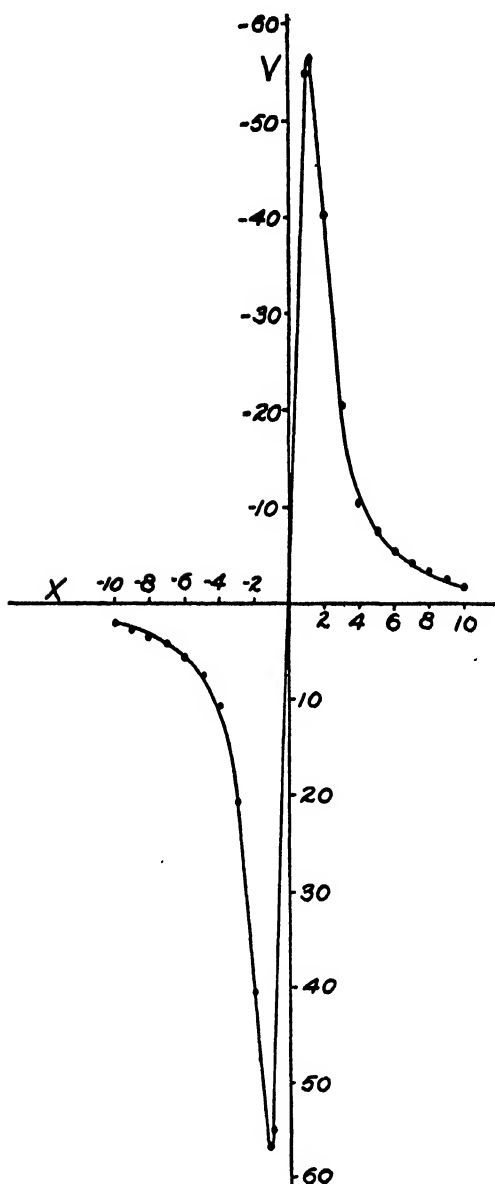


FIG. 4. Curve defined by equation (7) when  $c' = 100$ ,  $a = 1$ ,  $b = 1$ .

If we assume that the point  $P$  moves along the line  $y = b$  with a uniform velocity  $v$  from a point where  $x$  has a very large positive value to a point where  $x$  has a very large negative value, or that  $P$  is stationary and the origin moves from left to right with a similar

velocity we may replace  $x$  by  $vt$  where  $t$  is the time. In that case  $t$  must be measured in the same manner as  $x$ ; *i.e.*, it must be zero when  $x$  is zero, and negative when  $x$  is negative.

The interval that separates the positive and negative peaks of the experimental curve shown in Fig. 2 A is approximately 0.0036 second. Since the velocity of the excitation wave was about 1000 mm. per second, the distance  $2a$ , or the distance between the source and sink, assuming that  $b$  was very small, was in the neighborhood of 3.6 mm. This distance can, of course, be determined only approximately. The exact value of  $b$  is unknown, and furthermore, we have assumed that the region upon which the electrode rests may be regarded as a point. Actually, we are not measuring the potential at a point, but the mean potential, or something approaching this, of the region mentioned. With the soft-tipped electrodes that must be used, the size and shape of this region varies with different applications of the electrode, and, because of the movements of the auricle, to some extent from instant to instant. The size of the region over which the electrode and the auricular surface are in contact must clearly influence the form of the curve.

At any given instant the potential of the point under investigation must be the same, or nearly the same, as that of every other point that bears a similar relation to the crest of the wave of excitation. It is clear, therefore, that we must be dealing not with a single source and a single sink, but with a large number of sources and sinks, the former arranged along a line parallel to the wave crest and just in front of it, the latter along a similar line just behind it. It seems reasonable to suppose that there is a source and sink within each muscle fiber along which the excitatory process is passing. In that case equation (7) may be regarded as representing the potential variations produced at a given point by the activation of a single straight muscle fiber when  $b$  is the perpendicular distance from the fiber to the given point.

The experimental curve, on the other hand, is produced by the activation of a large number of muscle fibers, and is the algebraic sum of a large number of curves of the type defined by equation (7). For each of these curves  $b$  will have a different value; it is obvious, however, that those muscle fibers that are close to the electrode, for



which the value of  $b$  is small, will have individually a much greater effect upon its potential than the fibers that are more distant.

We have attempted to modify equation (7) in such a way as to include a large number of fibers. The expression

$$V = c' \mu \log_e \left( \frac{(\sqrt{(x-a)^2 + l^2 + b^2} + l) (\sqrt{(x+a)^2 + l^2 + b^2} - l)}{(\sqrt{(x-a)^2 + l^2 + b^2} - l) (\sqrt{(x+a)^2 + l^2 + b^2} + l)} \right) \quad (9)$$

defines the potential under the conditions postulated in Fig. 5, and the accompanying legend. The curve represented by this equation is shown in Fig. 6. It will be seen that the differences between this curve and the curve derived from equation (7) are of a minor kind.

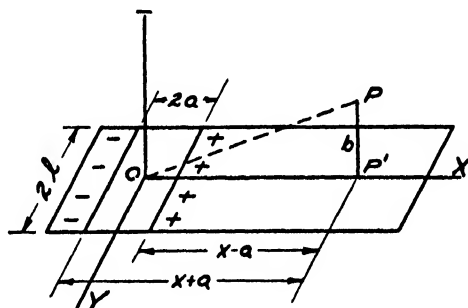


FIG. 5. The large rectangle lying in the  $XY$  plane and bisected by the  $X$  axis represents a thin sheet ( $2l$  units in width) of very long parallel muscle fibers. The number of fibers, and therefore the number of sources and of sinks, per unit width of the sheet is  $\mu$ . All of the fibers have been stimulated simultaneously and the crest of the excitation wave lies upon the  $Y$  axis. The distance between the sources and sinks that accompany this wave is  $2a$ . The exploring electrode is at the point  $P$ , of which the coordinates are  $x, o, b$ . The potential of the exploring electrode is given by equation (9) which is derived from equation (4).  $c'$  has the same significance in both equations.

In this case also the distance between the peak of maximum positivity and the peak of maximum negativity approaches  $2a$ , the distance between the source and sink produced by a single muscle fiber, as  $b$ , the distance from the electrode to the nearest muscle fiber, approaches zero.

Lewis and his associates (1914) have shown that the excitatory process, originating at the sinus node, spreads radially in all directions

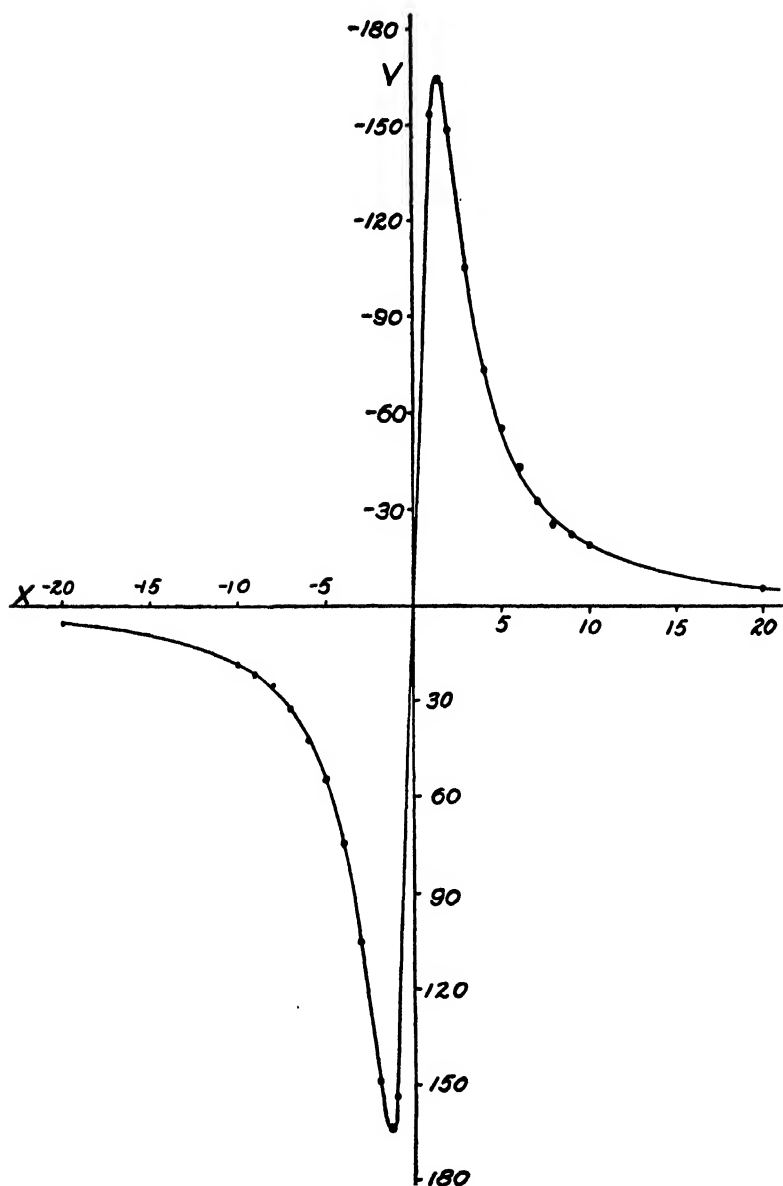


FIG. 6. Curve defined by equation (9) when  $c'\mu = 100$ ,  $a = 1$ ,  $b = 1$ ,  $l = 5$ .

with a more or less uniform velocity. Regarding the surface of the auricle as a plane, the crest of the excitation wave must lie at any instant upon the circumference of a circle. The sources and sinks

associated with this wave would be distributed somewhat as in Fig. 7 and the potential of a point near the auricular surface would be defined by the equation

$$V = \frac{4 c' \mu (x + a)}{\sqrt{(x + a + s)^2 + b^2}} \int_0^{\pi/2} \frac{d\phi}{\sqrt{1 - k_1^2 \sin^2 \phi}} - \frac{4 c' \mu (x - a)}{\sqrt{(x - a + s)^2 + b^2}} \int_0^{\pi/2} \frac{d\phi}{\sqrt{1 - k_2^2 \sin^2 \phi}} \quad (10)$$

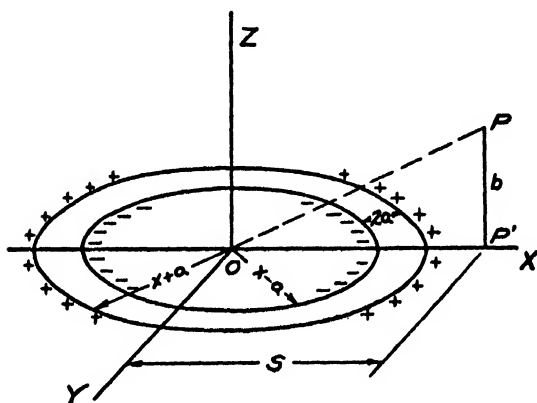


FIG. 7. The excitation wave has originated at the origin and is spreading in all directions with the same velocity through a thin sheet of muscle lying in the  $XY$  plane. The crest of this wave lies upon the circumference of a circle of radius  $x$ . The sources that precede it lie upon the circumference of a circle of radius  $x + a$ , and the sinks that follow it upon the circumference of a circle of radius  $x - a$ . The sources and sinks are equal in number and the number of the sources per unit length of arc is  $\mu$ . The exploring electrode is at the point  $P$ , of which the coordinates are  $s, 0, b$ . The potential of this electrode is given by equation (10).  $c'$  in this equation has the same significance as in equation (4). The two integrals are complete elliptic integrals, the values of which for known values of  $k$  may be found in suitable tables (*cf.* Pierce, 1910). The value of  $k_1$  is  $\frac{2\sqrt{(x + a)s}}{\sqrt{(x + a + s)^2 + b^2}}$  and the value of  $k_2$  is  $\frac{2\sqrt{(x - a)s}}{\sqrt{(x - a + s)^2 + b^2}}$ .

Here again  $b$ , the perpendicular distance from the given point to the auricular surface is introduced to avoid infinite values of the function and the distance between the peak of maximum positivity and the

peak of maximum negativity is approximately  $2a$  when  $b$  is very small.<sup>8</sup>

The type of curve defined by this expression is shown in Fig. 8. Unlike the curves shown in Figs. 4 and 6 it is asymmetrical. In general outline it is like the experimental curves obtained at relatively

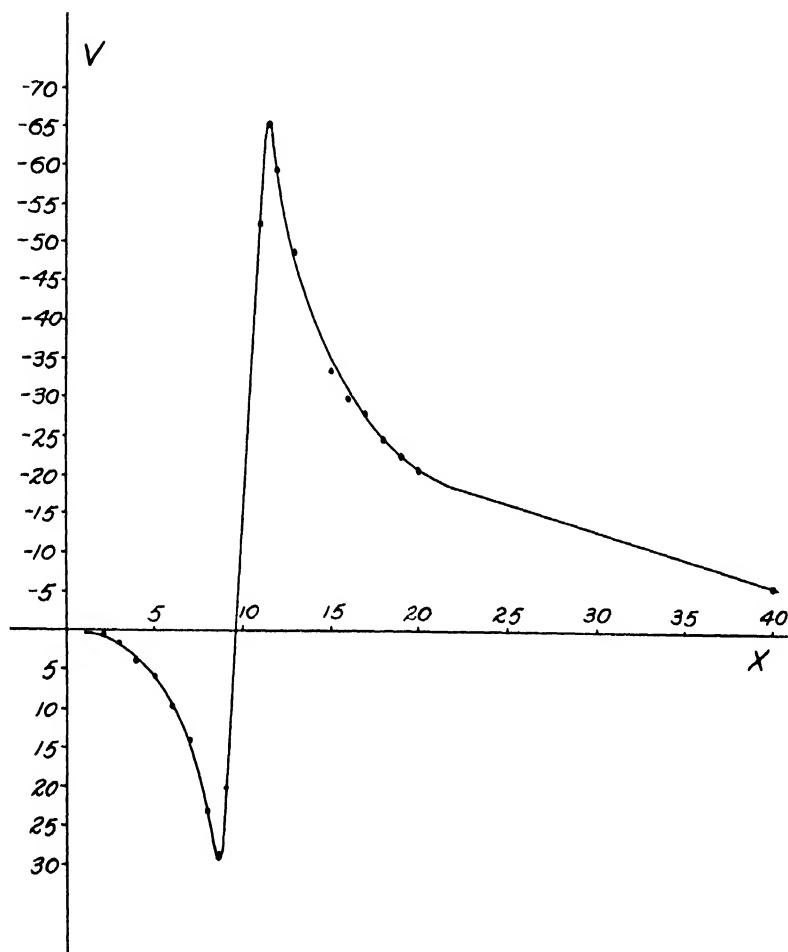


FIG. 8. Curve defined by equation (10) when  $4c'\mu = 100$ ,  $a = 1$ ,  $b = 1$ ,  $s = 10$

<sup>8</sup> In some respects, however, this last equation differs from the previous ones. Since  $x$  is the radius of a circle (Fig. 7) it cannot have negative values; furthermore, since the radius of the circle upon which the sinks lie is  $x - a$ , the equation becomes meaningless when  $x$  is equal to or smaller than  $a$ .

short distances from the sinus node (Fig. 9 A). When the exploring electrode is placed immediately over the node there is, of course, no downward deflection. As the electrode is moved away from the node a small downward deflection appears and increases in amplitude until the curve becomes nearly symmetrical. When the exploring electrode is placed near the tip of the auricular appendix the peak of maximum negativity is poorly developed (Fig. 9 B). At the extreme tip of the appendix it disappears. It is probable that the asymmetry of the curves obtained near the point where the excitation wave originates

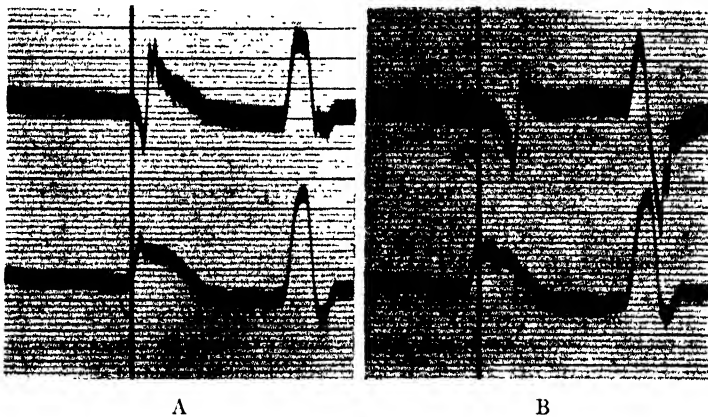


FIG. 9 A and B. Two curves from the same experiment as that shown in Fig. 2 A. The upper curves in A and B were taken in the same manner as the upper curve in Fig. 2 A, except that in A the exploring electrode was closer to the sinus node and in B it was near the tip of the auricular appendix. In A the intrinsic deflection begins 0.0125 second after the first auricular deflection in the standard lead and has a duration of 0.0046. In B the intrinsic deflection begins 0.0375 second after the first auricular deflection of the standard lead (lower curve) and has a duration of 0.0064 second.

and near the point where it is extinguished is due in part to the same circumstances that make the theoretical curve asymmetrical. When the distance of the electrode from either of these points is less than a wave length (the distance between the sources and sinks that accompany the fully developed excitation wave) the asymmetry is due chiefly to conditions that equation (10) does not take into account. For reasons that will become apparent later the sources and sinks grow in strength and separate as the excitation wave develops and

undergo the opposite changes when it is extinguished. A source of full strength cannot occur at a distance from the point of origin that is less than the full wave length; nor can a sink of full strength occur at a distance less than this from the point of extinction. Space is not available, however, for a complete discussion of this matter or of other factors that may make the experimental curve asymmetrical.

Regardless of which of the three equations is employed, the distance between the positive and negative peaks, when  $b$  is very small, is approximately  $2a$ , the distance between the source and sink assumed to be present within each muscle fiber along which the excitation wave is passing. It is suggested, therefore, that the interval which separates the peaks of the experimental curve obtained when a very small exploring electrode is placed in contact with the auricular surface at a point midway between the sulcus terminalis and the tip of the right auricular appendix is an approximate measure of the interval that separates the sources and sinks associated with the excitation of those fibers in close proximity to the electrode, providing that the arrangement of these fibers is not of too complex a kind and that all conduct with the same or nearly the same velocity. We may refer to this interval as the effective length of the excitation wave.

### *Electric Field of a Polarized Membrane*

The observations described suggest that the electrical effects produced by the excitation of a single muscle fiber are the same as those that would occur if the crest of the wave of excitation were preceded by a source and followed by a sink. The same conclusion can be derived on theoretical grounds from the so called membrane theory advanced originally by Bernstein which is too well known to require exposition here. We need merely say that according to this hypothesis each element of a resting muscle fiber is surrounded by a semi-permeable membrane and is in consequence the seat of a double layer of ions, a layer of cations, to which the membrane is permeable, on its outer surface and a layer of anions, to which the membrane is impermeable, on its inner surface.

Under these circumstances physiologists are accustomed to speak of the membrane as polarized (Bayliss, 1918, pp. 649-650). It is held that when a muscle element passes from the resting to the active

state the membrane bounding it becomes permeable to the anions as well as to the cations and is depolarized; when it returns to the resting state it is repolarized.

Since the membrane is supposed to be very thin and since the cations on its outer surface are positively and the anions on its inner surface negatively charged, a polarized membrane is equivalent electrically to a thin polarized shell. The potential  $V$  at any point  $P$  of an infinite homogeneous medium<sup>9</sup> within which such a membrane is immersed is determined by the expression

$$V = \phi \omega \quad (11)^{10}$$

where  $\phi$  is a constant,<sup>11</sup> which may be defined as the electrical moment of a membrane of unit area, and  $\omega$  is the solid angle subtended by the membrane at  $P$ ; *i.e.*,  $\omega$  is the area cut out upon a spherical surface of unit radius inscribed about  $P$  by the cone formed by drawing lines from  $P$  to every point upon the boundary of the membrane. When an observer who is stationed at  $P$  and is looking through this cone sees the positive side of the membrane,  $V$  is positive; when he sees the negative side of the membrane,  $V$  is negative. It is convenient to consider the solid angle that determines  $V$  to have the same sign.

By the boundary of the membrane is meant an edge at which it terminates. In this sense a tennis ball has no boundary but if it be cut in two parts (equal or unequal) each portion has a boundary which is the cut edge. When a part of the membrane is depolarized the boundary is the line which separates the polarized from the depolarized part.

The solid angle subtended by the completely closed polarized shell or membrane that surrounds a resting cell (Fig. 10) at any point outside the cell ( $P_0$ ) is zero (Pierce, 1902, p. 215). Consequently, the potential at all points outside a resting muscle fiber immersed in an infinite homogeneous conductor would be zero. The solid angle

<sup>9</sup> The assumption that the medium, instead of being infinite, is a sphere of large radius does not materially alter the character of the electric field.

<sup>10</sup> Cf. Pierce, 1902, 215.

<sup>11</sup> Equation (11) is derived from equation (5) of Fig. 1.  $\phi$  equals  $2ac'\mu$ , where  $a$  and  $c$  have the same significance as in that equation and  $\mu$  is the number of sinks or negative charges on the inner and the number of sources or positive charges on the outer surface of the shell or membrane per unit area.

subtended at any inside point ( $P_I$ ) is  $4\pi$  hence the potential at all points inside the fiber would be  $4\pi\phi$  and would be negative since the negative charges are on the inner surface of the polarized membrane. In passing across the membrane from an outside to an inside point there would be a sudden drop in potential amounting to  $4\pi\phi$ ; a discontinuity of the potential function similar to that which occurs in an electric cell. So long as the polarization of the membrane remained unaltered, the drop in potential across it would be the same whether it formed an open or a closed surface (Pierce, 1902, p. 215), *i.e.*, if

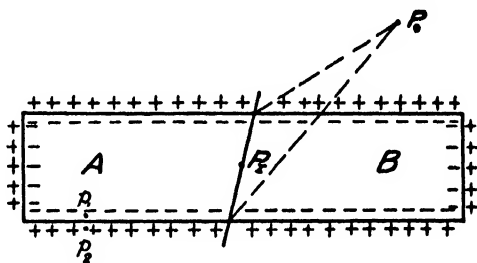


FIG. 10. The diagram represents a cylindrical cell surrounded by a polarized membrane and immersed in an infinite conducting medium.  $P_o$  is any point external to the cell and  $P_I$  any point inside the cell. A plane (represented by a line) through  $P_I$  divides the closed membrane that surrounds the cell into two parts A and B, each of which may be considered alone as if the other were not present. The solid angle subtended at  $P_o$  by A, that portion of the membrane lying to the left of the dividing plane, is defined as the area cut out upon a spherical surface of unit radius inscribed about  $P_o$  as center by a certain cone. This cone is formed by drawing lines (indicated by broken lines) from  $P_o$  to every point upon the boundary of A. When an observer stationed at  $P_o$  looks through this cone he sees the negative side of the membrane A, and the solid angle subtended by this membrane at  $P_o$  is therefore negative. The boundary of B is the same as the boundary of A (the boundary of each membrane being the edge that abuts upon the plane through  $P_I$ ), and the solid angle subtended by B at  $P_o$  is equal to that subtended by A at the same point in absolute magnitude. Since an observer who is looking through the specified cone sees the positive side of B, the solid angle subtended at  $P_o$  by this membrane is positive. Consequently, the solid angle subtended at  $P_o$  by A and B together is zero. The solid angle subtended by A at  $P_I$  and that subtended by B at the same point are each equal to  $2\pi$ , and both are negative. The lines drawn from  $P_I$  to the boundary of A and B lie in the plane passed through this point. This plane may be considered a cone whose vertex is at P and whose solid angle is  $2\pi$ .

$p_1$  and  $p_2$  are two points very close together, one on either side of the membrane A.



$p_1$  and  $p_2$  (Fig. 10) are two points very close together, but on opposite sides of a completely closed and uniformly polarized membrane the difference in potential between them ( $4\pi\phi$ ) will not be altered by removing or depolarizing a distant portion of the membrane so long as the charges upon that portion of the membrane that lies between and adjacent to them are not disturbed.

The maximal electromotive force across a semipermeable membrane polarized in the manner specified is given (Donnan, 1924-25) by the equation

$$E = \frac{RT}{nF} \log_e \frac{c_1}{c_2} \quad (12)^{12}$$

where  $R$  is the gas constant expressed in electrical units;  $T$ , the absolute temperature;  $F$ , the Faraday equivalent;  $n$ , the valency of the diffusible cation; and  $c_1/c_2$  the ratio of the concentration of this cation on one side of the membrane to its concentration on the other. Consequently,

$$\phi = \frac{RT}{4\pi nF} \log_e \frac{c_1}{c_2} \quad (13)$$

Inasmuch as the solid angle subtended by a polarized membrane is not determined by the configuration of the membrane but solely by its boundaries, the electrical fields produced in an infinite medium by two membranes, polarized in the same sense and to the same intensity, which have the same boundaries must be identical. In Fig. 11 the polarized disk  $A$  and the polarized membrane  $B$ , a cylindrical shell open at one end, taken together form a completely closed surface. The potential at  $P$ , any point outside this surface, due to  $A$  and  $B$  taken together is therefore zero. We may regard this potential as the sum of two potentials,  $V_A$ , the potential at  $P$  due to  $A$  alone, and

<sup>12</sup> This is the Nernst equation for a concentration cell. It has been found that the electromotive force is determined by the ratio of the activities of the ion (Lewis and Randall, 1923) rather than by the ratio of its concentrations. In dilute solutions these two ratios do not differ materially. This equation is introduced here to show that the constant  $\phi$  has the dimensions of an electromotive force, and to indicate the nature of the factors that may determine its value. Whether this equation, which is reasonably accurate in the case of non-living systems, can be applied to living cells is for the purpose of this article immaterial.

$-V_B$ , the potential at  $P$  due to  $B$  alone. We have then  $V_A + (-V_B) = 0$ ; consequently  $V_A$  and  $V_B$  are equal in magnitude. If we reverse the charges on  $A$ , as in Fig. 11,  $b$ , the solid angles subtended by  $A$  and  $B$  at  $P$  which are equal in magnitude will have the same sign and the former may be substituted for the latter in computing

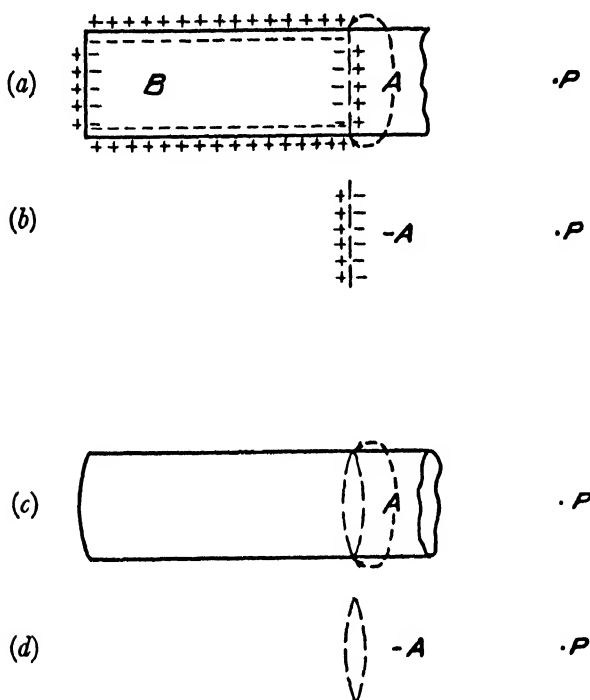


FIG. 11. The diagram  $a$  represents a cylindrical cell, surrounded by a membrane which is represented by a continuous line. That portion of the membrane that is represented as extending for an indefinite distance to the right of the broken line  $A$  has been completely depolarized. The remainder of the membrane, labelled  $B$ , is still polarized. The transition from the depolarized to the polarized portion of the membrane is represented as abrupt.  $P$  is any point outside the cell. In order to compute the effect of the polarized membrane  $B$  upon the potential at  $P$  we imagine a polarized disk  $A$ , indicated by a broken line, placed at the junction of the polarized and depolarized portions of the cell;  $A$  and  $B$  together will then form a closed polarized surface, and the potential at  $P$  due to  $A$  and  $B$  together must be zero. The effect of the polarized membrane  $B$  upon the potential at  $P$  must, therefore, be the same as the effect produced by the polarized disk  $A$  with the polarity of the charges reversed as in diagram  $b$ . This is merely a mathematical method of obtaining a convenient resultant of the charges upon the membrane  $B$ . In this sense and in this sense only  $-A$  may be substituted for  $B$ .

the potential of points external to the closed surface which they form when taken together.

It is clear that if the excitation of a muscle fiber consisted in the instantaneous and complete depolarization of the membrane bounding successive elements of the fiber, so that an abrupt transition occurred at the junction of the polarized and depolarized portions of the membrane, the electric field at outside points due to the charges upon the former and the electric field that would be produced by the charges upon a polarized disk located at this junction would be identical. The disk must be polarized to the same intensity as the membrane of the resting fiber and the negative side of the disk must face toward the active muscle. Under these circumstances a polarized disk traveling along the fiber would give all of the electrical effects associated

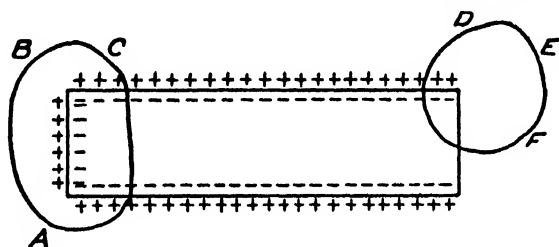


FIG. 12. The diagram represents a cylindrical cell surrounded by a polarized membrane except that the disk of membrane that closes one end of the cylinder has been completely depolarized. *ABC* is any circuit that does not pass and *DEF* any circuit that does pass through the depolarized portion of the membrane.

with the wave of excitation and at any instant the electric field would be such as might be produced by polarization of the surface separating the active from the resting muscle.

In so far as these conclusions are concerned the extent of the conducting medium in which the muscle is immersed is immaterial. The electromotive force across the membrane must be the same whether the medium is limited or infinite; it is defined by equation (12) into which the extent of the medium does not enter. Imagine that it were possible to completely depolarize the membrane at one end of a muscle fiber without altering the other charges (Fig. 12). Let *ABC* represent any possible circuit in the medium that does not pass through the depolarized portion of the membrane. If this circuit cuts the polarized membrane it must do so an even number of times and the sum of

all the potential discontinuities that it contains must be zero. *DEF* represents any circuit that passes through the depolarized membrane at the end of the fiber and crosses the polarized membrane an odd number of times. The sum of the potential discontinuities in this circuit must be  $4\pi\phi$ . If the depolarized portion of the membrane is now repolarized no current will flow in any circuit. It is a general principle that if in any circuit or in any network, containing any number of batteries, the addition of another battery reduces the currents flowing to zero, the currents in all circuits will be the same as they were originally if the polarity of the last battery is reversed and all others are removed.<sup>13</sup> Whatever the extent of the medium in which the fiber is immersed, the electric field of the partially depolar-

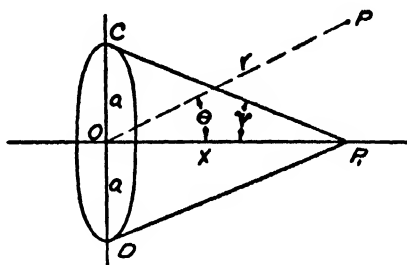


FIG. 13. *CD* is a disk of radius  $a$ , whose center is at  $O$ .  $P_1$  is any point on the axis of this disk,  $P$  any point not on the axis.

ized fiber must, therefore, be equivalent to that of the circular disk of polarized membrane polarized in a sense opposite to that required to reduce the current in all circuits to zero.

The potential at any point in an infinite homogeneous medium in which a polarized disk is immersed is determined by the solid angle subtended by the disk at that point. In Fig. 13 the magnitude of the

<sup>13</sup> It is, of course, required that these procedures shall not alter the resistances of the circuits involved. In applying this principle of substitution to the partially depolarized muscle fiber, it should be remembered that any peculiarities of resistance must not be changed. If the membrane, the interior of the cell, and the medium differ in conductivity the polarized disk that is substituted for the charges distributed over the partially depolarized fiber will not be immersed in a homogeneous medium; the resistance after substitution *must* be the same as before.

solid angle subtended by the disk  $CD$  at a point on its axis ( $P_1$ ) is defined by the equation

$$\omega = 2\pi (1 - \cos \psi) \quad (14)$$

In order to define the magnitude of the solid angle subtended at a point not on the axis an infinite series is required.<sup>14</sup> If the radius of the disk is small, that of a muscle fiber, the solid angle subtended by it at any relatively distant point  $P$  is, however, very nearly equal to  $\frac{S \cos \theta}{r^2}$  where  $S$  is the area of the disk and  $r$  and  $\theta$  have the significance indicated in Fig. 13. If the disk is polarized the potential at  $P$  will be defined approximately by the equation

$$V = \phi S \frac{\cos \theta}{r^2} \quad (15)$$

This expression is identical with that which defines the potential at any point due to a single source and a single sink lying very close together upon the axis of the disk and equidistant from its center. The strength of this doublet is proportional to the product of the area of the disk and the intensity to which it is polarized. The magnitude of the electric currents produced at a given instant by a partially depolarized muscle fiber should likewise be proportional to the product of its cross-sectional area and the intensity of its polarization.

### *Gradual Depolarization*

In the foregoing discussion it has been assumed for the sake of simplicity that the activation of a muscle fiber is accompanied by complete and instantaneous depolarization of each successive portion

<sup>14</sup> If the disk  $CD$  were polarized the potential at any point  $P$  (Fig. 13) would be exactly defined by the series

$$V = \phi S \frac{\cos \theta}{r^2} - \phi \pi \left[ \frac{1.3}{4} \frac{a^4}{r^4} P_3(\cos \theta) - \frac{1.35}{4.6} \frac{a^6}{r^6} P_5(\cos \theta) \dots \text{etc.} \right]$$

in this expression  $S$  is this area of the disk and  $P_m(\cos \theta)$  is the appropriate coefficient of Legendre. This series is convergent if the ratio of  $a$  to  $r$  is less than 1. It will be noted that equation (15) may be derived from this series by dropping all terms except the first.

of the fiber. So long as all portions of the fiber are depolarized to the same extent it will not matter whether depolarization is complete or incomplete. Obviously, instantaneous depolarization is impossible, and we must therefore attempt to determine to what extent the effects produced by gradual depolarization will differ from those that would occur if depolarization occurred instantaneously.

If the number of charges per unit surface in the resting fiber is  $\mu$  the distribution of the charges may be considered equivalent to  $N$  distributions of density  $\frac{\mu}{N}$ ; *i.e.*, we may conceive of the total charge

as made up of  $\frac{\mu}{N}$  layers of infinitesimal thickness, each layer containing

$\frac{\mu}{N}$  charges per unit surface. Suppose that depolarization of the fiber begins at one end; that complete depolarization has taken place to the right of  $A$  in Fig. 14; and that between  $A$  and  $B$  partial depolarization has taken place, so that the number of charges per unit surface increases progressively from  $A$  to  $B$  where the intensity of polarization becomes that of the resting fiber. Let that portion of the cylindrical fiber which lies between  $A$  and  $B$  be divided into  $N - 1$  zones by means of  $N$  thin laminae perpendicular to the axis of the cylinder. The first of these laminae will pass through  $A$ , the  $N$ th through  $B$ . Suppose that  $N$  is a very large number and consequently that the zones are so narrow that the number of charges per unit surface within any zone is constant; the number of charges per unit surface will then be  $\frac{\mu}{N}$  over the first zone,  $2\frac{\mu}{N}$  over the second zone,  $3\frac{\mu}{N}$  over the third, and so forth, reaching a value of  $\frac{\mu(N - 1)}{N}$  in zone  $N - 1$ . If

the circular disk cut out of each lamina by the curved surface of the cylinder is polarized to density  $\frac{\mu}{N}$ , the positive charges being toward the depolarized end of the muscle, the distribution will be equivalent to  $N$  completely closed surfaces over each of which the polarization density is  $\frac{\mu}{N}$  and the potential at any point outside the cylinder due to this distribution will be zero. Consequently, the potential at any point

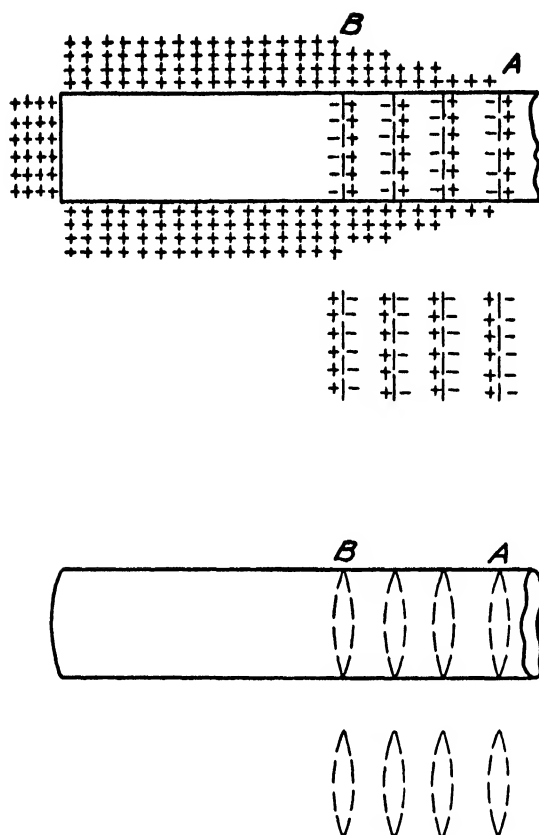


FIG. 14. The diagram represents a cylindrical cell surrounded by a membrane (indicated by a continuous line). This membrane is uniformly polarized to the left of *B* (to avoid confusion only the positive charges are indicated) and completely depolarized to the right of *A*. Between *B* and *A* the intensity of polarization declines by successive steps to zero. In order to determine the effect of this polarized membrane upon the potential of any point outside the cell we imagine a number of polarized disks (indicated by broken lines) located between *A* and *B*. Each disk is placed at a point where a drop in the intensity of polarization occurs and forms with the layer of sources and sinks (only the former are shown) that does not extend to the right of it a completely closed and uniformly polarized surface. The potential at any point outside the cell due to the polarized membrane and the polarized disks taken together will therefore be zero. Consequently the polarized membrane will have the same effect upon the potential at an outside point as would be produced by the disks if the polarity of their charges were reversed as indicated. In this sense the polarized disks may be substituted for the polarized membrane in computing the electric field outside (but not inside) the cell.

outside the cell due to the charges distributed over the partially depolarized muscle fiber will be the same as the potential at the same point due to the  $N$  polarized disks lying between  $A$  and  $B$  with the polarity of the charges reversed as indicated in Fig. 14. Since there are  $N$  disks distributed over the distance  $AB$  which we may call  $L$ , the mean number of disks per unit length within the interval  $AB$  is  $\frac{N}{L}$ . Providing that the number of disks per unit length is the same for all parts of  $AB$ , the potential at  $P$  due to all of the disks will be defined by the expression

$$V = \frac{\phi S}{L} \left( \frac{1}{PB} - \frac{1}{PA} \right) \quad (16)$$

Consequently, the potential at  $P$ , any point outside the cell, is equivalent to that produced by a single source at  $B$  and a single sink at  $A$ ; that is to say, a source at the point where the depolarization is just beginning and a sink at the point where it has just become complete. The potential of an electrode placed very close to the muscle fiber will reach its maximum positivity at the moment when depolarization of the subjacent membrane begins, and its maximum negativity at the moment when depolarization of the subjacent membrane is complete.

If the distance  $AB$  is small in comparison with the distance of  $P$  from the fiber the above equation becomes

$$V = \frac{\phi S}{L} \left( \frac{L \cos \theta}{r^2} \right) = \phi S \frac{\cos \theta}{r^2}$$

and if this equation is compared with equation (15) it will be seen that the potential of an electrode distant from the fiber will be the same as it would be if depolarization of each successive element of the fiber took place instantaneously.

In the above discussion it has been assumed that the rate of depolarization is constant; in other words, that after depolarization of any given portion of the membrane begins, it proceeds at a uniform rate until complete. It is very improbable, however, that depolarization takes place in that way. Suppose that it proceeds very slowly at first, then with increasing rapidity until its rate is maximal, and finally



with diminishing speed until it is complete. The mean number of polarized disks, or sources and sinks, per unit length within the interval  $AB$  will still be  $\frac{N}{L}$ ; but the number of disks in any given subdivision  $\Delta L$  of this interval will vary with the position of  $\Delta L$  within the interval. If  $\Delta L$  is near the beginning or end of the interval the number of sources and sinks will be small; if it is near the middle of the interval the number will be large. The distribution of sources and sinks will be similar to that shown in Fig. 15 in which each square represents a source and sink, or a polarized disk. In this curve the ordinate at any given point represents merely the number of sources and sinks per unit length of the interval  $AB$  at the corresponding point within the interval; all of the sources and sinks are supposed to lie upon the line  $AB$ . Each horizontal row of squares may be represented by a single source and a single sink as indicated in the figure. For the

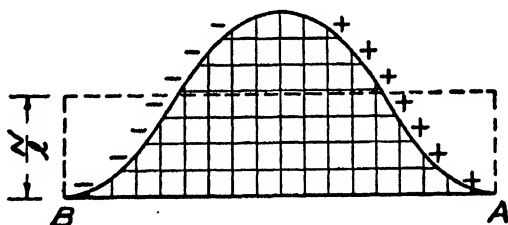


FIG. 15. At a given instant the membrane that surrounds a cylindrical cell is uniformly polarized to the right of  $A$  and completely depolarized to the left of  $B$ . Between  $A$  and  $B$  it is partially depolarized. The rate at which the intensity of polarization is changing at any point between  $A$  and  $B$  is proportional to the length of the ordinate that passes through that point. For the purpose of determining the potential at any point outside the cell we may substitute for the positive and negative charges that remain upon the polarized and partially depolarized portions of the membrane a certain distribution of charges obtained by placing within each element of the interval  $AB$  the number of polarized disks indicated by the ordinate of the curve passing through that element. These polarized disks, each of which is approximately equivalent to a source and sink very close together (a doublet) are indicated by small squares. They are proportional in number to the area under the curve. For these disks we may in turn substitute the sources and sinks indicated by appropriate signs, all of which are supposed to lie upon the line  $AB$ . If  $l$  represents the length of the line  $AB$  and  $N$  the total number of polarized disks, the mean number of disks per unit length of  $AB$  will be  $\frac{N}{l}$  and the area under the curve will be equal to that of the rectangle indicated.

lowest row of squares the source will be at *A* and the sink at *B*. For the other rows the source and sink will be closer together and both will lie between *A* and *B*. Inspection of the figure shows that the concentration of sources will be greatest at the point where the rate of depolarization is increasing most rapidly, and that the greatest concentration of sinks will occur at the point where the rate of depolarization is decreasing most rapidly. We may therefore expect that if this distribution of sources and sinks passes along a muscle fiber with a uniform velocity, an electrode placed close to the fiber will display maximum positivity approximately at the time when the rate of depolarization of the membrane beneath it is increasing most rapidly, and maximum negativity at the time, approximately, when the rate of depolarization of the membrane beneath it is decreasing most rapidly.<sup>15</sup> The distance between the peak of maximum positivity and the peak of maximum negativity in such curves as that shown in Fig. 2 *A* must depend upon the form of the curve that represents the rate of depolarization at a given point.

The total number of sources and sinks within the distribution is represented by the area under the curve (Fig. 15), which is equivalent to the area of the rectangle of which *AB* forms one side and  $\frac{N}{L}$ , the mean number of sources and sinks per unit length of *AB*, the other. At points distant from the muscle in comparison with the length *AB* the electrical effects will be the same whatever the distribution of sources and sinks within the interval *AB*. Consequently, it will be immaterial whether the rate of depolarization is constant or variable, or whether depolarization at a point takes place gradually or instantaneously.

### *Repolarization*

When the period of activity comes to an end, and the muscle fiber is restored to the resting state, repolarization of the membrane is assumed to take place. If the order of repolarization is the same as the order of depolarization the electrical effects produced by it must

<sup>15</sup> This conclusion holds only in case the curve that represents the rate of depolarization belongs to a certain class.

be opposite in polarity to those produced by depolarization. With this exception all of the statements made with respect to depolarization apply with equal force to repolarization. The form of the curve that represents the potential variations produced by the repolarization of a single muscle fiber at a point near the fiber will depend upon the distance  $AB$ , where  $A$  is the point at which repolarization is just beginning and  $B$  the point at which it has just become complete; upon the form of the curve that represents the rate of repolarization at any given point; and upon the velocity of the wave of repolarization along the muscle fiber. At points distant from the fiber in comparison with the length  $AB$  the potential at any instant will be the same as if repolarization at any given point took place instantaneously.<sup>16</sup> It is clear that if the order of repolarization is the same as the order of depolarization the total electrical effect produced by the one process must be equal in magnitude but opposite in sign to that produced by the other. In other words, the total quantity of electricity flowing through any given circuit must be the same in both cases.

We should expect the curve shown in Fig. 2 A to be followed by a curve of similar form in which the positions of the positive and negative phases were reversed. This portion of the curve, however, is hidden by the occurrence of ventricular deflections. Although we have performed a few experiments in which the production of complete heart block prevented interference of this kind, we are not as yet in a position to offer a satisfactory analysis of the electrical effects produced at points upon the auricular surface by repolarization of the auricular muscle. It is obvious, however, that repolarization takes place very slowly in comparison with depolarization and that the former is modified by a great many factors that have little or no effect upon the latter. Consequently, the number of fibers or units producing electric forces simultaneously is much greater in the case of repolarization than in the case of depolarization and the order of the two processes is not necessarily the same.

<sup>16</sup> This statement does not apply during the period when the recovery wave is developing or being extinguished; nor does the similar statement made with reference to depolarization apply when the excitation wave is developing or being extinguished.

*Comments*

In the preceding pages we have described briefly certain curves obtained by leading directly from the mammalian auricle, and have attempted to analyze these curves by applying the principles that govern the distribution of electric currents in volume conductors. Our analysis involves certain simplifying assumptions that are not strictly in accord with the facts. The equations and conclusions based upon these assumptions are, therefore, to be regarded as approximations only.

We have attempted to develop a mathematical theory that would explain the observations relating to the distribution of the action currents produced by excitable tissues immersed in or in contact with a large body of conducting material made by ourselves and by others. We have employed the language of the so called membrane theory and have founded the mathematical treatment of our problem upon its postulates. It should be pointed out, however, that the distribution of the electric currents under consideration must be essentially the same regardless of the manner of their origin.

Perhaps this statement should be amplified by a few words of explanation. From the mathematical standpoint an electromotive force existing at a boundary may be represented by a polarized surface. If there is a difference of potential between the interior and the exterior of a resting cell and this difference of potential decreases or disappears when the cell is activated, the electric field will be the same whatever the mechanisms involved may be.

Where the electromotive force does not arise at a single boundary, but within a transitional zone which may be considered an infinite succession of boundaries, each marking an infinitesimal physico-chemical change, the matter within this zone may be regarded as electrically polarized. The essential characteristic of the theory presented is that it defines the electric field about a muscle fiber undergoing activation as equivalent to that which would be produced by polarization of those portions of the fiber that are passing from the resting to the active state or *vice versa*. It is required that the effective electric forces shall be confined, or shall appear to be confined, within these regions; the mechanism of their production is immaterial. It is clear, so it seems to us, that this requirement must be met by

any hypothesis that seeks to explain the origin of the action current. The polarity of the electric forces associated with excitation and of those that accompany recovery is determined by the relative positions of the active and resting portions of the muscle fiber. This is a plain indication that no unbalanced electric forces exist except as a consequence of the presence of zones within which excitation or recovery is in progress. These are the only portions of the fiber, so it would seem, within which there is a gradient that can determine polarity. A muscle fiber in the resting state or in the same state of activity throughout its length can hardly produce an electric current when it is completely surrounded by a homogeneous medium. In either case the boundaries that define the different phases responsible for the heterogeneous character of the tissue are completely closed, and any electric forces that exist at these boundaries must be in equilibrium. There is nothing in the situation to distinguish one end of the fiber from the other; no unbalanced polarity that can become evident external to the tissue.

The membrane theory has the important advantage that it explains the current of action and the current of injury on the same basis. According to its postulates the effective electric forces responsible for the current that accompanies excitation do not actually arise within the transitional zones referred to, but only appear to do so because of the disturbance within these regions of a previously existing equilibrium.

It must be borne in mind that in writing the equations that define an electric field it has been assumed that the electric conductivity is uniform throughout all space, *i.e.*, that the medium, the membrane, and the interior of the cell have the same conductivity, and also that the conductivity of the membrane is not altered by depolarization. It is improbable that there is any tissue that meets these requirements, and these equations cannot be expected to be exact in a quantitative sense. In the case of cardiac muscle they appear to represent actual conditions with sufficient accuracy to be useful, and it seems probable that they will hold reasonably well for skeletal muscle and possibly nerve.

The equations mentioned are also based upon the assumption that the medium is infinite. The distribution of the action current will,

however, be the same in all essential particulars, if the excitable tissue is deeply embedded in any extensive medium.

#### SUMMARY

The action currents produced by heart muscle and other tissues immersed in or in contact with a large body of conducting material are distributed in accordance with the laws that govern the flow of electric currents in volume conductors.

The curve obtained when one electrode (the exploring electrode) is placed very close to and the other (the indifferent electrode) very far from the active tissue may be regarded as representing the potential variations of the exploring electrode alone; the potential of the indifferent electrode is by comparison nearly constant.

Curves obtained by this method of leading from the surface of the mammalian auricle indicate that the electrical effects produced by the passage of the excitation wave along a single muscle fiber are nearly the same as those that would occur if the crest of this wave were immediately preceded by a source and followed by a sink.

A study of the electric field of a polarized membrane immersed in a volume conductor shows that this conclusion may be derived on theoretical grounds from the membrane theory of Bernstein.

We wish to thank Dr. H. B. Williams and Dr. Kenneth Cole for a careful and critical reading of the first draft of this article with particular reference to the mathematical processes and the physical and physiological principles involved, and also for the valuable suggestions that they have given us.

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## ELECTROKINETIC PHENOMENA

### X. ELECTRIC MOBILITY AND CHARGE OF PROTEINS IN ALCOHOL-WATER MIXTURES

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#### I

#### INTRODUCTION

The following experiments were undertaken with the object of studying the surface properties and ionization of adsorbed proteins in alcohol-water mixtures.<sup>1</sup> When the charge on the protein as measured by titration curves is compared with the surface charge on the adsorbed protein as measured by the mobilities of protein-covered quartz particles, there is obtained information about the effect of the medium on the dissociation of the protein, the orientation of polar groups, and the relationship between charge and electrophoretic mobility.

Gelatin was used because it has been shown (1) that under the conditions maintained here, mobilities of gelatin and of deaminized gelatin (both adsorbed onto quartz) are in the same ratio as the respective amounts of acid (base) bound. This means that in aqueous media gelatin obeys the rule (1) that in solutions of the same ionic strength the electrophoretic mobility of the same protein at different hydrogen ion activities should be proportional to the number of hydrogen or hydroxyl ions bound by each molecule. It should be noted that this rule can apply to the adsorbed gelatin only if the active (dissociating) groups are free, that is oriented toward the liquid. Further, the rule will apply only if the protein salt is equally dissociated over the range of hydrogen ion activity considered.

<sup>1</sup> It has been shown that in general the mobilities of freely dispersed proteins are equal to the mobilities of particles coated with the same proteins.



The rule just stated results from the Debye-Henry approximation (equation (8), reference (1)),

$$Q = 6 \pi \eta r v_m (\kappa r + 1), \quad (1)$$

where  $v_m$  = electrophoretic mobility =  $\mu/\text{sec.}/\text{volt}/\text{cm.}$ ,  $r$  = radius,  $Q$  = charge,  $\eta$  = viscosity,  $\kappa = \sqrt{\frac{4 \pi e^2}{D k T} \sum n_i z_i^2}$ . For a given molecule in a given medium this becomes  $Q = v_m (C' + C'')$ , (equation (8a), reference (1)). But when the medium is altered (keeping the ionic strength constant),  $Q$  becomes a function of  $v_m$ ,  $\eta$ , and  $D$ . This provides a test of the question whether in equation (1) the viscosity and the dielectric constant of the medium can be used to predict changes in  $Q$ . Since the medium was altered by the addition of ethyl alcohol it was not possible to vary the viscosity and the dielectric constant independently, but the combined effect was readily investigated.

The main part of the work was the study of the mobilities of gelatin in 35 per cent and in 60 per cent ethyl alcohol for comparison with each other and with titration curves in like concentrations of alcohol. In connection with this a shift of the isoelectric point of gelatin in the presence of ethyl alcohol was found and this shift was studied. But before any mobility measurements could be made it was necessary to study the change in electrophoretic velocity with change in field strength in alcohol solutions.

## II

### *Methods*

The pH measurements of solutions of alcohols other than ethyl alcohol were made with a hydrogen electrode referred to the pH of N/10 HCl as 1.07. The remaining pH measurements were made with a quinhydrone electrode. This can be used for ethyl alcohol since no reaction occurs between quinhydrone and alcohol-water, but a correction is necessary<sup>2</sup> (2). The accuracy of the time measurements in electrophoresis obtained here is such that the average deviation is

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<sup>2</sup> The quinhydrone electrode was tested against a hydrogen electrode in a number of ethyl alcohol solutions; the two agreed to better than 0.05 pH.

The titration of gelatin in alcohol was checked with the glass electrode. The writer is indebted to Prof. Hans Clarke for permission to use the facilities of his laboratory.

usually less than 5 per cent. Exceptions occur when very low velocities are measured either very near the top or the bottom of the cell, or when a very low voltage is used. All other errors were well within these limits. The temperatures at which the experiments were done were always recorded, but the variations from 20° were not sufficient to make worth while corrections for temperature, except of course in the pH determinations.

The mobility measurements were made in the modified Northrop and Kunitz microelectrophoresis cell described by Abramson (3). The theory of von Smoluchowski (4) was followed. The field strength was found from the cross-section of the cell, the current flowing, and the conductivity of the solution, as follows:

$$\frac{\text{Current in amperes}}{\text{Sp. conductance} \times \text{cross section in cm.}^2} = \text{volts/cm.} = X.$$

The suspensions were made up in the following order: quartz, protein solution (in the case of gelatin sufficient to make one part in a thousand), acid or buffer, alcohol and water. Cooper's gelatin was used. The expression "per cent alcohol" is used to mean ml. of alcohol per 100 ml. of solution. The quartz used was a carefully purified suspension. The gelatin solutions were made by heating, but never above approximately 40° (5), to get rid of the air bubbles formed on mixing alcohol with water, and then cooled to room temperature.

The dielectric constants used are those given by Wyman (6) for pure alcohol-water mixtures, uncorrected for the salt and protein. The solutions contained one part in a thousand of gelatin; since 5 gm. of gelatin per 100 gm. of mixture lower the dielectric constant from 81 to 68 (7), the error here is negligible.

### III

#### *Electrophoretic Velocity and Field Strength*

The characterization of particles by the measurement of mobilities depends on the experimental fact that electrophoretic velocity is proportional to the field strength. Theoretically, the right hand side of the equation for the mobility of a small particle (10),

$$v_m = \frac{v}{X} = \frac{D\zeta}{6\pi\eta}, \quad (2)$$

(where  $v$  = electrophoretic velocity,  $\zeta$  = electrokinetic potential,  $X$  = field strength), is constant when  $X$  is varied. If this were not so experimentally the mobility would have to be measured as a function of one or more variables and the interpretation would be greatly complicated. The linear relationship has been found generally (11), with few exceptions.

Recently, however, the results of Ettisch and Zwanzig (12) and of Martin and Gortner (13) have suggested that in alcoholic solutions  $\zeta$  is a function of the velocity, while Köhler (14) has found that in non-alcoholic solutions the volume outflow in electroosmosis is not proportional to the field strength. Ettisch and Zwanzig studied streaming potentials. They used very dilute sodium chloride solutions and found that with the pure solution  $\zeta$  was independent of pressure, but that in the presence of methyl alcohol  $\zeta$  increased with increasing pressure. For equal pressures and varying alcohol concentration  $\zeta$  went through a minimum. Propyl alcohol at high concentrations even reversed  $\zeta$ . Köhler used a palmitic acid diaphragm and measured the rate of outflow of electrolyte solutions under the influence of varying current. In none of these cases is there any definite independent proof of laminar flow.

Traube and Whang (15) and Traube and Dannenberg (16) showed that water flowing through a glass capillary coated with a polar substance (such as a fatty acid) flowed much faster than through the uncoated tube, when the angle at which the tube was tipped was small (about  $3^\circ$ ); that is, when the pressure was low. Also the outflow speed of water solutions of surface active substances such as alcohols was greater than for pure water flowing through the same capillary, but again at a small angle only. At a greater angle (under greater pressure) both effects disappeared.

Because of these recent suggestions that  $v/X$  may not always be constant and because of the flow anomalies (15) at low velocity gradients, it was considered necessary to demonstrate that under our conditions  $v/X$  is constant.

As can be seen from Figs. 1 and 2, the field strength in these experiments with protein-covered<sup>3</sup> quartz particles in various alcohol-water mixtures was varied from 2 to 20-fold. It is definitely shown that for field strengths of the order of magnitude of those used in the experiment, the mobility of these proteins when adsorbed on quartz in these alcohols is not a function of the field strength.

<sup>3</sup> By suitable experiment it was demonstrated that gliadin is adsorbed by quartz, forming a complete film.

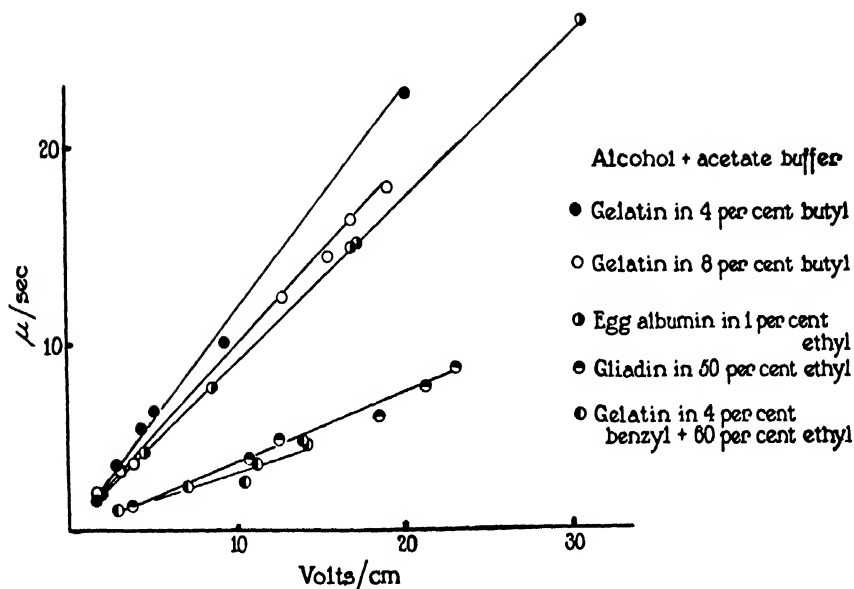


FIG. 1. The electrophoretic velocities of quartz particles coated with various proteins in various alcohols are plotted against the field strength. For each protein in a given medium the velocity is proportional to the field strength.

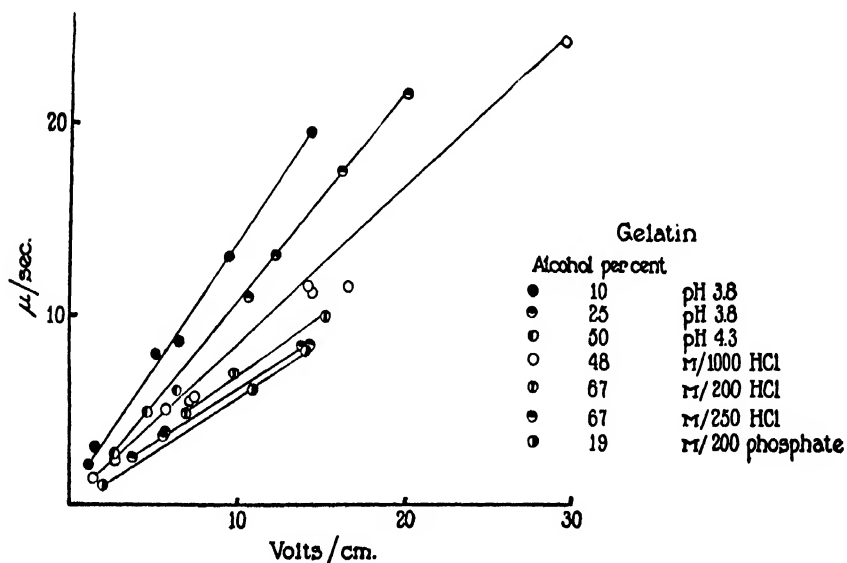


FIG. 2. The electrophoretic velocities of gelatin-covered quartz particles in media containing various percentages of ethyl alcohol are plotted against the field strength. In each medium the velocity is proportional to the field strength.

## IV

*Electrophoresis and Electroosmosis*

It has previously been found that inert particles covered with proteins have, in general, mobilities independent of the bulk radius of curvature of the particle (8). That is, the same electric mobility is obtained in aqueous media whether the inert particle is microscopic in size or is very large. That this is also true for protein surfaces in alcohols has been found here by studying the ratio of electrophoretic to electroosmotic mobility. Fig. 3 is for gelatin surfaces in 40 per

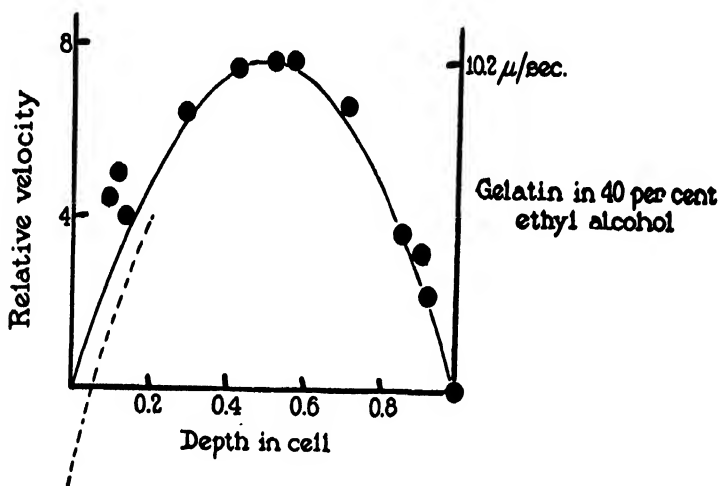


FIG. 3. The relative velocities of gelatin-coated quartz particles in 40 per cent ethyl alcohol at different depths in the electrophoresis cell are plotted against depth in the cell. The full line is the parabola  $y = -30.4x^2 + 30.4x$ . The fact that the points fall on a parabola going through the origin is evidence of the equality of the electrophoretic and electroosmotic velocities.

cent ethyl alcohol. Similar results were obtained in several trials with gliadin. The dotted line is the curve on which the points should fall according to the theory of Debye (9), according to which electrophoretic and electroosmotic mobilities are not equal. Evidently, however, the electrophoretic and electroosmotic mobilities are equal, and in the experiments to be discussed the size of the quartz particles used does not influence the mobilities obtained.

## V

*Electric Mobilities of Gelatin in Alcohol-Water Mixtures*

Fig. 4 shows the mobilities of gelatin-coated quartz particles in  $N/150$  sodium acetate buffer in 0 per cent, 35 per cent, and 60 per cent ethyl alcohol. It is clear that alcohol shifts the isoelectric point of the gelatin toward smaller hydrogen ion activities. It is also obvious that alcohol lowers very greatly the maximum mobilities. This lowering combined with the shift in isoelectric point causes the curves to intersect.

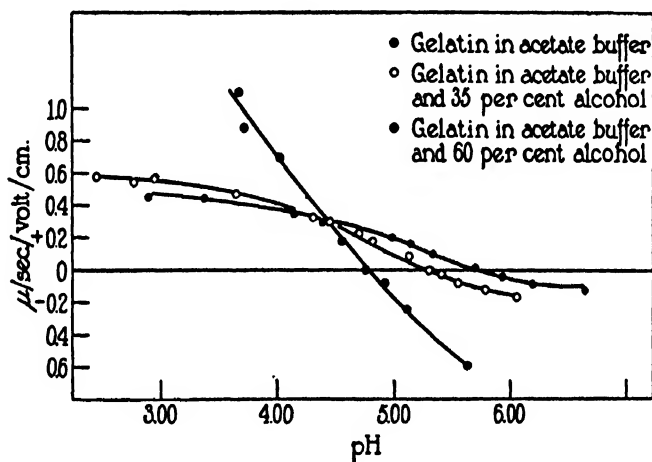


FIG. 4. The electrophoretic mobility of gelatin-covered quartz particles is plotted against the pH of the medium for media containing different percentages of ethyl alcohol. In the more acid regions NaCl-HCl mixtures were used in place of the acetate buffers.

The lowering of the mobilities by alcohol is not a simple phenomenon. Alcohol changes both the dielectric constant and the viscosity of the medium and may also be expected to alter the electrokinetic potential. According to equation (2) each of these three changes will alter the mobilities.

Further corrections for these changes are not entirely straightforward. The question has frequently been raised (17) whether the dielectric constant and viscosity of the medium or the quite different values which might be expected to obtain within the double layer should be substituted in equation (2). Here values for the medium in bulk will

be used. In the following section it will be possible to show that these values of  $D$  and  $\eta$  can be used in combination to predict changes in charge from mobilities.

At this point those differences in the mobilities which were due to altered viscosity were eliminated by calculating a quantity called here corrected mobility;

$$\text{corrected mobility} = v_m \eta / \eta_0,$$

where  $\eta_0$  = viscosity of pure water. This quantity has the significance that the differences between curves of corrected mobilities should be due to changes in the dielectric constant alone, the values being in

TABLE I

$V_m$			$V_m \eta / \eta_0$		
0 per cent alcohol	35 per cent	60 per cent	0 per cent	35 per cent	60 per cent
0.20	0.08	0.06	0.20	0.20	0.16
0.40	0.15	0.10	0.40	0.40	0.30
0.60	0.21	0.15	0.60	0.54	0.42
0.80	0.25	0.19	0.80	0.67	0.54

The figures in each horizontal row are for pH's of equal charge as determined by the titration curves.

some ways more representative of the effect of the alcohol itself on  $v_m$ .

As mentioned previously, the data in Fig. 4 give the mobilities uncorrected for  $\eta$ . It would involve too many complications to discuss the change in  $v_m \eta / \eta_0$  except near the isoelectric point, where the curves are nearly linear over a small distance. In Table I there are compared values obtained from smoothed curves of  $v_m$  and  $v_m \eta / \eta_0$  for equal charge (as determined by the amount of acid bound). The large differences in  $v_m$  disappear almost completely when the correction for  $\eta$  is applied, only a slight decrease taking place as the alcohol concentration increases. This result is similar to Walden's results for ions (18).

## VI

*Mobilities and Titration Curves*

Before proceeding to a comparison of titration and mobility curves in solutions of different dielectric constant, the rule following from equation (1) was tested in 35 per cent alcohol and again tested in 60 per cent alcohol. If  $\eta$ ,  $r$ , and  $\kappa$  are constant it follows from equation (1) that  $Q \propto v_m$  and also that  $Q \propto$  corrected mobility. In any one concentration of alcohol and at constant ionic strength  $\eta$ ,  $r$  (19), and

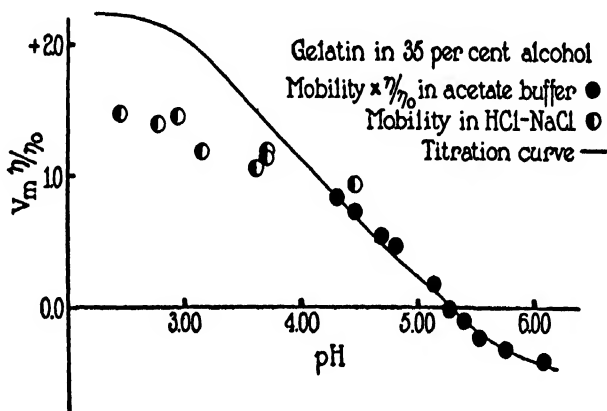


FIG. 5. The circles represent the corrected mobility (as this is defined in the text) of gelatin-covered quartz particles in 35 per cent ethyl alcohol from pH 2.5 to pH 6.00. The full line is the titration curve of gelatin in 35 per cent alcohol. The arrangement of the scale of the titration curve is explained in the text.

$\kappa$  will be constant. Since on the assumptions (1) underlying the rule given in Section I,  $Q$  is the number of hydrogen or hydroxyl ions bound by a molecule,  $Q$  is proportional to the mols of acid (base) bound by a gram of gelatin and is represented by the titration curve. Thus acid bound and corrected mobility will be proportional to one another if the rule is obeyed.

The titration curves were constructed on the assumption that no acid or base is bound at the isoelectric point. Fig. 5 shows  $v_m \eta/\eta_0$  in 35 per cent alcohol plotted with the titration curve in 35 per cent alcohol in such a way that the two curves coincide at two points.



That is, the scales are arranged so that the two curves correspond at pH 4.30 as well as at the isoelectric point. Fig. 6 shows  $v_m \eta/\eta_0$  and titration curve in 60 per cent alcohol similarly arranged with pH 2.94 chosen for correspondence. Figs. 5 and 6 show that in each medium considered separately the mobility is proportional to the combining power, and hence presumably to the charge, in 60 per cent alcohol and up to pH 4.0 in 35 per cent. The discrepancy of about 30 per cent in the very acid region in 35 per cent alcohol may be due to the fact that the mobilities were determined in solutions of constant, the titration curves of varying, ionic strength. As a result of the as-

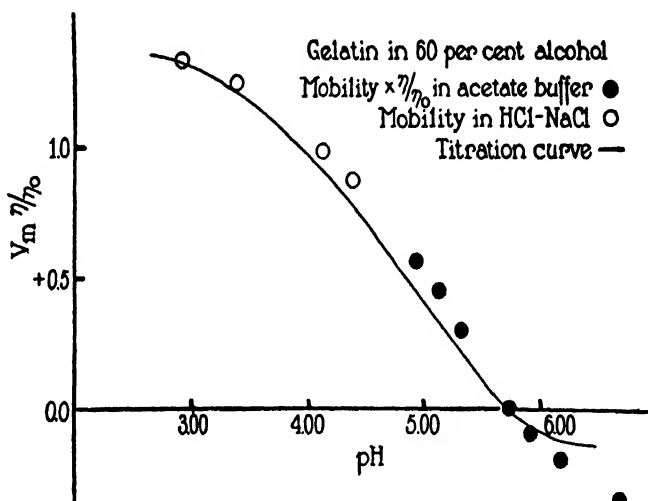


FIG. 6. The circles represent the corrected mobility (as this is defined in the text) of gelatin-covered quartz particles in 60 per cent ethyl alcohol. The full line is the titration curve of gelatin in 60 per cent ethyl alcohol. The arrangement of the scale of the titration curve is explained in the text.

sumptions underlying the rule given in Section I the proportionality makes it possible to conclude that in alcohol-water mixtures as well as in the previously investigated water solutions gelatin is adsorbed with the polar groups oriented toward the liquid. It is interesting that in 60 per cent alcohol the portions of both the mobility and the titration curves which lie near the isoelectric point were determined with partly or completely precipitated gelatin, yet both curves are smooth.

## VII

*Electric Mobility, Titration Curve, and Charge*

By comparing the mobilities in the different media (differing in dielectric constant and viscosity) it is possible to test to some extent the applicability of the viscosity and the dielectric constant of the bulk of the medium to the electrophoresis equation for charge, equation (1). The simplest means of doing this is to calculate charge from mobility by means of equation (1), using the viscosity and dielectric constant of the bulk of the medium. If now the acid bound (measured directly), which on the assumptions referred to above is proportional to the charge on the protein, is, in different media, in the same ratio as the charge calculated from the mobility by equation (1), then within the limits of the experimental error equation (1) may be used to predict changes in charge, using the viscosity and dielectric constant of the bulk of the medium.

In the calculation of the charge from equation (1) certain complications arise. The use of the factor  $6\pi$  in equation (2) is based on the fact that the mobilities of certain dispersed proteins and of protein-covered particles have been found to be equal (17). Under these circumstances the radius to be used will be not that of the quartz particle but that of the protein molecule. For gelatin  $2 \times 10^{-7}$  cm. was used, from the molecular weight (20). However, had  $1 \times 10^{-7}$  cm. or  $3 \times 10^{-7}$  cm. been used, the final conclusions would not have been noticeably different, although the absolute values of the charges would have been altogether different.

Fig. 7 shows the agreement between  $Q$  from equation (1) and titration curves in the middle pH region for 0 per cent alcohol and 35 per cent. This graph was made by drawing the 0 per cent alcohol titration curve and charge points to scales which made them coincide and then drawing the 35 per cent titration curve and charge points to the same scales. All the charge points calculated from mobilities determined in acetate buffer fall very well onto the titration curve. The charge points calculated from mobilities determined in NaCl-HCl do not agree so well. The first of these points is shown in Fig. 7. This point indicates the general divergence from theory. That there should

be irregularities connected with the change from acetate buffer to a NaCl-HCl mixture is not surprising, since the ionic type as well as the valence does exert an influence (21).

For the more acid regions of the 35 per cent curve, as just pointed out, and for the 60 per cent curve, the agreement is less complete (Fig. 8). Because of slight disagreement between theory and experiment except under optimal conditions with 35 per cent alcohol and acetate buffers (Fig. 7), all of the data for acetate buffers and NaCl-HCl mixtures from pH 2 to pH 7 have been plotted as in Fig. 8,

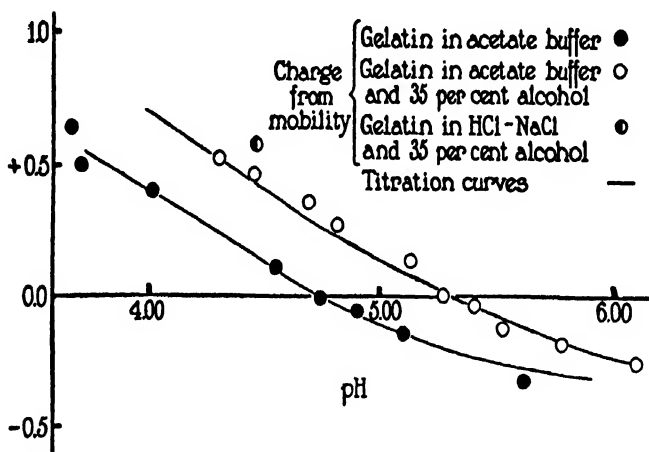


FIG. 7. The full circles show the charge of gelatin calculated from the mobility of gelatin-covered quartz particles in acetate buffer. The open circles show the charge calculated from the mobility in acetate buffer and 35 per cent ethyl alcohol. The lines are titration curves of gelatin in 0 per cent and in 35 per cent ethyl alcohol. The figure is limited to a range fairly close to the isoelectric point.

titration curves in the upper half, mobility curves in the lower. The titration curves of gelatin in 0 per cent and 35 per cent alcohol have been compared from pH 2 to pH 10. The curves are very much of the same shape, the isoelectric point being shifted to a higher pH, the curves converging at the limits. Gelatin, therefore, becomes a weaker acid in alcohol.

The disagreement in the more acid region of the 35 per cent charge and titration curves when plotted to the same scales as the 0 per cent curves is probably due to several causes. First is the previously men-

tioned shift from acetate buffer to NaCl-HCl solution. Second is the fact that since the mutual action of the ions may be greater in the acid range, it is likely to be even more so in alcohol in the acid range. This would lead to the charge calculated from the mobilities being in the very acid region too low for the titration curve arranged as previously explained, and this seems to be the case.

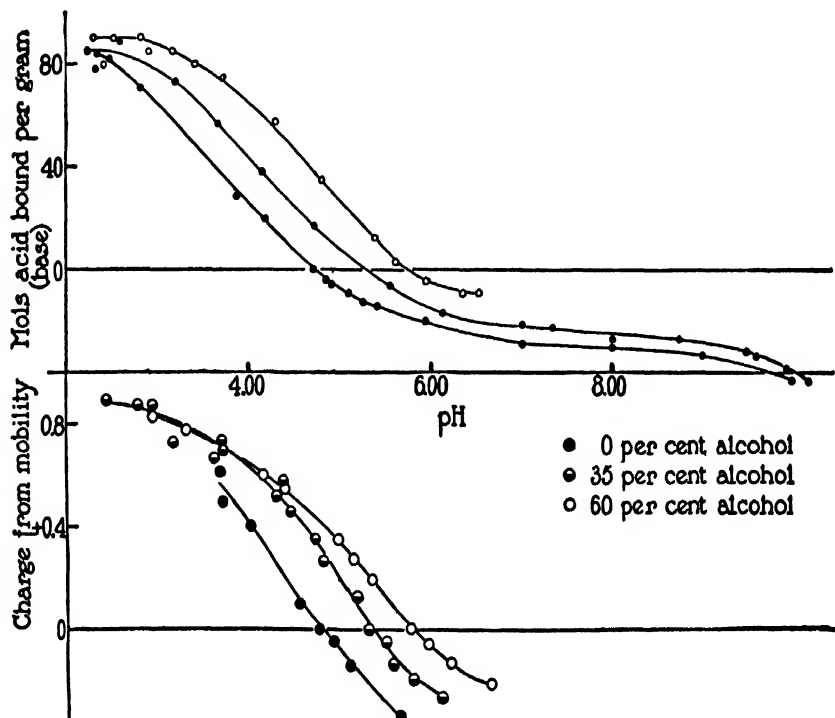


FIG. 8. The upper curves are titration curves of gelatin in 0 per cent, 35 per cent, and 60 per cent ethyl alcohol. The lower curves are the charge curves calculated from the mobility of gelatin-covered quartz particles in 0 per cent, 35 per cent, and 60 per cent ethyl alcohol, the circles being experimental points.

Dehydration by the alcohol probably affects chiefly the results in 60 per cent alcohol (22-24). This conclusion is consistent with the fact that going from 0 per cent alcohol to 35 per cent, acid bound and charge calculated from mobility are proportional, while the proportionality does not extend to the gelatin in 60 per cent alcohol.

However, the agreement with theory in the case of the change from

0 per cent to 35 per cent alcohol points to the complete ionization of the protein salt in alcoholic solutions in the neighborhood of the isoelectric point (since it is completely ionized in aqueous solutions), and to the applicability of the ordinary dielectric constant and viscosity, and the usefulness of the Debye-Henry equation, in the prediction of changes of charge.

## VIII

*Isoelectric Point*

The isoelectric point of the gelatin-covered particles was taken by interpolation from the smoothed pH mobility curves or determined from experiments arranged to show no motion in the electric field. In the presence of alcohol the isoelectric point of particles covered with gelatin films was found to be shifted toward smaller hydrogen ion activities as shown in Fig. 9. The shift is related apparently linearly to the volumes per cent of alcohol in the solution and also linearly to the dielectric constant (neglecting the salt) of the solution. The form of the relationship will be discussed later. The direction and order of magnitude of the shift must first be accounted for.

Michaelis and Mizutani (25) measured the pH of a very dilute mixture of equivalent amounts of amino acid and the sodium salt of the amino acid in alcohol solution (at various concentrations of alcohol). The hydrogen ion activity (referred to the normal hydrogen electrode in pure water and neglecting liquid junction potentials) of this solution they called  $k_2$ . The constant similarly measured in acid solution they called  $k_1$ . Making the assumption that the ratio of the activity coefficients of the protein anion and cation remains constant for any one concentration of alcohol, it follows that the hydrogen ion activity of the isoelectric point is  $\sqrt{k_1 k_2}$ . Then,

$$\text{pH of the isoelectric point} = -1/2 \log k_1 k_2 = 1/2 \text{p}k_1 + 1/2 \text{p}k_2,$$

and

$$\Delta \text{pH of the isoelectric point in going from one alcohol concentration to another} = 1/2 \Delta \text{p}k_1 + 1/2 \Delta \text{p}k_2.$$

Of course gelatin is not monobasic monoacidic, nor are its first acidic and basic constants exactly equal to those of glycoll. Nevertheless

since Michaelis and his coworkers found the effects of alcohol on various organic acids to be much the same, it is possible to estimate from

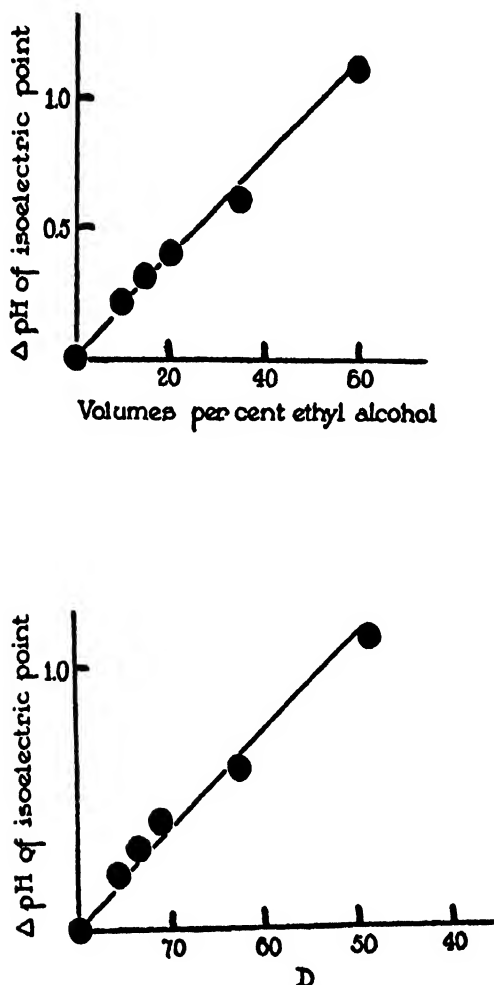


FIG. 9. Above, the change in pH of the isoelectric point of gelatin, caused by ethyl alcohol, is plotted against the volumes per cent alcohol in the solution. Below, the same data are replotted as change in pH of the isoelectric point against dielectric constant of the solution.

the glycooll results at least the direction and order of magnitude of the change to be expected in the case of gelatin.

$$\Delta \text{pH} = 1/2 \Delta \text{p}k_1 + 1/2 \Delta \text{p}k_2 = (\text{for glycooll going from 0 per cent to 60 per cent alcohol}) 0.42.$$

This is in the same direction and is of the same order of magnitude as the shift of 1.1 found experimentally for gelatin. Using the values for para-aminobenzoic acid

$$\Delta \text{ pH (from 4 per cent to 60 per cent) } = 0.93.$$

The form of the shift, which is linear with the dielectric constant, is interesting. Abramson<sup>4</sup> has found that if the isoelectric points in alcohol solutions are calculated from the dissociation constants obtained by Michaelis and Mizutani for a number of amino acids, the isoelectric point is in each case linearly related by a limiting law to the dielectric constant of the medium (if the salt is neglected). This is true both for glycocoll and for the aminobenzoic acids. The fact that similar changes are produced by alcohol for gelatin and for simple ampholytes is in harmony with our present notions of the simple amphoteric behavior of proteins.

#### SUMMARY

1. The electrophoretic velocities of gelatin-, egg-albumin-, and gliadin-covered quartz particles in various alcohol-water solutions are, within the limits employed in usual experimental procedures, proportional to the field strength.

2. The electrophoretic mobilities of small, irregularly shaped quartz particles covered with an adsorbed film of protein in alcohol-water solutions are equal to the electroosmotic mobilities of the liquid past similarly coated flat surfaces. Hence the size and shape of such particles does not influence their mobilities, which depend entirely on the protein film.

3. The corrected mobility and hence presumably the charge of gelatin-covered quartz particles in solutions containing 35 per cent ethyl alcohol is proportional to the combining power of the gelatin; therefore the gelatin is adsorbed with the active groups oriented toward the liquid. The same is true in 60 per cent alcohol.

4. The charge calculated by means of the Debye-Henry approximation from the mobility of gelatin in solutions containing up to 35 per cent ethyl alcohol is, in the neighborhood of the isoelectric point, pro-

<sup>4</sup> Work not yet published.

portional to the combining power of the gelatin. Therefore the dielectric constant and the viscosity of the bulk of the medium may be used in the Debye-Henry approximation

$$Q = 6 \pi \eta r v_m (1 + \kappa r)$$

to predict changes in charge from mobility.

5. In the neighborhood of the isoelectric point gelatin is probably completely ionized in buffered ethyl alcohol-water mixtures up to 60 per cent alcohol.

6. In the presence of ethyl alcohol the isoelectric point of gelatin is shifted toward smaller hydrogen ion activities. This shift, like that caused by alcohol in the isoelectric points of certain amino acids, is approximately linearly related to the dielectric constant of the medium.

The writer is greatly indebted to Dr. Harold A. Abramson, who suggested this work and under whose direction it was carried out.

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# ON THE RATE OF OXYGEN CONSUMPTION BY FERTILIZED AND UNFERTILIZED EGGS

## IV. CHAETOPTERUS AND ARBACIA PUNCTULATA

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In three earlier papers (18-20) measurements have been given of the rate of oxygen consumption by the fertilized and unfertilized eggs of the brown alga *Fucus vesiculosus*, the clam *Cumingia tellinoides*, and the polychaete worm *Nereis limbata*. This paper presents the results of similar although more extensive measurements on the eggs of the annelid *Chaetopterus*, and measurements on the eggs of the sea urchin *Arbacia punctulata*. The measurements on these several forms have all been made in absolute units, as a function of the volume of (wet) eggs, and a comparison of the rates on the same absolute scale shows some order and correlation between the changes in rate which separately appear wholly diverse. The comparative relations can best be considered after the results of the measurements on *Chaetopterus* and *Arbacia* have been given, and they are discussed in Paper V (21).

The eggs of the *Chaetopterus* are a little more than 100 microns in diameter. They are obtainable in abundance in good seasons, such as during the months of July and August, 1930, when these measurements were made at Woods Hole. Enough eggs were obtained from one female for a complete experiment with controls. Warburg manometers were used. Of the species mentioned above, the *Chaetopterus* eggs are preeminently the most suited to manometric measurements. They withstand shaking to an exceptional degree, with comparatively little effect on the rate of oxygen consumption, the fertilization, or the development. The

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\* National Research Council Fellow in the Biological Sciences when most of this work was carried out.

eggs are enclosed in a tough outer membrane which lifts at fertilization to become the fertilization membrane, as may be demonstrated with the microdissection needle. The presence of this tough membrane on the unfertilized egg is no doubt partly responsible for the resistance of the egg to damage from shaking. The eggs of *Nereis* and *Cumingia* also have tough protecting membranes and they too are comparatively constant in their metabolic rates and other activities during the course of prolonged shaking. The relatively naked unfertilized eggs of *Fucus* and *Arbacia* on the other hand are so susceptible to too rapid shaking, which causes an increase in the respiratory rate and then cytolysis, that results are more variable. It is difficult to avoid depression of the rate of respiration by  $\text{CO}_2$  retained in the medium if the rate of shaking is too greatly reduced in order to avoid damage to these eggs, while on the other hand an erroneously high measure results if the rate of shaking is so fast as to damage the eggs, and this is the error which is most apt to occur unless care is taken.

The proper rate of shaking of the manometers is a complex function when limitations are imposed by delicacy of the biological material. The rate must be rapid enough to maintain gas equilibrium between the sea water and the gas space in the vessel and will therefore depend both on the concentration of eggs and upon their absolute rate of respiration. Preliminary experiments were made with *Chaetopterus* eggs, using the results of previous work already done on *Fucus* as a starting point, to determine the best rate of shaking with the vessels employed. This was followed by a biological check. First, the least approximate concentration of eggs was determined which would consume enough oxygen in a short time to shift the manometer fluid well beyond the limits of the reading error. With approximately this concentration of eggs as a working basis, the absolute rate of oxygen consumption per unit volume of eggs was measured with changes in the rate of shaking. The condition of the eggs after prolonged shaking at various rates was observed and samples were inseminated. The rate of oxygen consumption was the most sensitive of these factors to the rate of shaking. With the particular vessels employed, and with an amplitude of 7.5 cm., it was found that thirty round trip shakes per minute or less gave a reduced rate of respiration compared with higher shaking rates, and further, a rate which steadily declined as consecutive measurements were made. With from thirty-six to forty-eight shakes per minute however the absolute rate was the same, and neither increased nor decreased when consecutive measurements were made for as long as 8 hours. It seemed safe then to assume that forty-eight round trip shakes per minute was rapid enough to avoid suppression, and further it did not damage the eggs in respect to any feature affecting respiratory rate, since this rate did not change with prolonged shaking (Table I). Unfertilized eggs shaken steadily at this rate for 8 hours repeatedly fertilized in the same proportion as fresh controls at the start of the experiment, often 95-100 per cent fertilization. Fertilized eggs shaken for 8 hours developed as uniformly and at the same rate as unshaken controls in

covered finger-bowls. In all experiments the shaking was more than adequate to keep the eggs uniformly distributed throughout the medium.

For special purposes it was sometimes advisable to increase the concentration of eggs, *e.g.* in order to take more frequent readings with measurable consumptions of oxygen. In general an increase in concentration of eggs requires a greater rate of shaking, unless there is already a good margin. Most of the measurements were made with egg concentrations ranging from 1 part in 25 to 1 part in 100 by volume of sea water. Throughout this range forty-eight shakes per minute gave similar absolute measurements of rate. At this rate of shaking however 1 part of eggs in 5 of sea water yielded a measure which was approximately half the standard absolute rate, and 1 part in 8 about 65 per cent. These low rates also continued to drift lower with time, indicating that gas equilibrium was not being maintained

TABLE I

*Consecutive Hourly Measurements of the Rate of Oxygen Consumption by Unfertilized Chaetopterus Eggs in an 8 Hour Run. A Typical Good Experiment*

Hours	Rate (mm. <sup>3</sup> O <sub>2</sub> per hour per 10 mm. <sup>3</sup> eggs)
1	2.4
2	2.5
3	2.5
4	2.4
5	2.4
6	2.4
7	2.3
8	2.4

even at this fairly rapid rate of shaking, which was entirely adequate for lesser egg concentrations. One of the reasons for avoiding high concentrations of eggs is that they require high rate of shaking which damages the eggs.

The measurements were made in a thermostat at 21°C.  $\pm$  0.05. Four Warburg manometers with rectangular vessels were used in each experiment. These were designed to hold a large fluid volume compared with the gas volume in order to increase sensitivity of measurement and make it possible to use comparatively dilute egg concentrations. These vessels were approximately 40 mm. long, 15 mm. wide, and 11 mm. high, inside dimensions, with wells for KOH and bulbs on the side to hold sperm suspensions or KOH. The manometer capillaries were small, 0.6 mm. in diameter, to lessen the gas volume, and especially that part of it outside the thermostat and therefore not subject to temperature control. In the four vessels the total working volumes were 8.62, 8.95, 8.96, and 9.01 cc. In the experiments either 2 cc. or usually 4 cc. of sea water or egg suspension were placed in the vessels, giving in the latter case a fluid-gas volume ratio of about

4:5. This increased the sensitivity of measurement to about five times that of some of the standard conical types of vessels. In the rectangular vessel it is possible to increase the depth of fluid without decreasing the area of gas contact as in the conical vessel. The depth of fluid was approximately 3.5 mm. when the vessels contained 2 cc., and twice as deep when the vessels contained 4 cc.

In the typical experiment, one vessel contained sea water and served as a barometric control, two contained unfertilized eggs, and the fourth contained sea water to which sperm were added in the same amount and at the same time that one of the egg containing vessels was inseminated. Just prior to the addition of sperm suspension a like volume of water was withdrawn in order to keep the volume of solution constant. This fourth vessel served as a control for the respiration of the spermatozoa. In all cases this was found to be negligible; *i.e.*, of the order of the limits of measurement or less. Each of the vessels contained 0.2 or 0.4 cc. 5 per cent KOH solution in the well or bulb to absorb  $\text{CO}_2$ . After measurements had been made of the respiratory rate of the unfertilized eggs, taken consecutively every 10, 15, or 20 minutes for more than an hour, or until a series of concordant values had been obtained, one of the vessels was inseminated. The other egg-containing vessel served as an unfertilized control. In a number of cases this unfertilized control was also inseminated later, so that it came to be a second measurement, but with any errors due to environmental variables shifted to a different time period after insemination compared with the first vessel inseminated.

### *The pH of the Medium*

The 5 per cent KOH solution in the well absorbs  $\text{CO}_2$  from the gas space, and therefore tends to shift the buffer system in the alkaline direction. Some time before the measurements on the *Chaetopterus* eggs were made a few blank runs were made with sea water and with tap water in conical vessels with indicator dyes. In both a slow but steady alkaline shift occurred. In the sea water the pH determinations were only approximate as there was a salt error. After an hour of shaking the pH of the sea water was between 8.4 and 8.6. After 2½ hours it had become about 8.8; after 4 hours about 9 or 9.2. After 4 hours shaking was stopped but the vessels remained unopened overnight and the next day after a total of 23 hours, it was of the order of 9.8. In another similar experiment after a total of 20 hours it was about 9.5.

It is obvious however that with tissues in the medium which are producing  $\text{CO}_2$  the alkaline drift will be retarded if not completely neutralized or reversed. It will not be reversed, if the shaking maintains gas equilibrium, so long as the KOH solution is unsaturated and has adequate surface exposure. For reasons already considered it seems safe to assume that suppressing acidity was avoided by empirically finding the adequate rate of shaking for the concentration of eggs used. Many of my measurements lasted only about 2 hours, and in these had there been no compensatory  $\text{CO}_2$  production whatsoever the pH might have been raised to around 8.7 or 8.8, and in the 8 hour experiments to above 9. It is well established

that alkalinity tends to increase respiratory rate, although the effect is not great until pH 9.5 or 10 is approached. In fact, however, due to egg production of  $\text{CO}_2$  this alkaline drift must have been considerably retarded. It is hardly to be supposed that the compensation was exact so that no change of pH took place, although the compensation was probably quite close. It would be possible, by special means, to keep exact measurement of the pH during an experiment, but this seems not very necessary in view of the biological check which exists. Since repeated consecutive measurements for 8 hours (the longest runs made) on unfertilized eggs, which have a high rate of respiration, showed constant respiratory rate, it appears that under the conditions pH change was not great enough in either direction to change the respiratory rate measurably. Neither was it great enough to alter the fertilizability or the development of the eggs.

### *Preparation of the Eggs*

A large fresh female *Chaetopterus* was placed in a finger-bowl of sea water and the tip of each parapodium was cut off with a pair of scissors. More than  $\frac{1}{4}$  cc. of eggs then exuded into the sea water. The female was removed, and debris was taken out with forceps. A certain amount of mucous remained with the eggs but this was removed by at least three thorough washings of the eggs, the sea water being pipetted off after the eggs had settled to the bottom. Care was taken not to remove so much sea water as to compress the eggs on the bottom. Pouring the water off was also avoided for the same reason. This washing process took about an hour. In no case were eggs used which had not been in sea water for an hour. These eggs are immature when shed, but as soon as they come into sea water maturation processes proceed to the metaphase of the first polar spindle. The eggs rest in this stage indefinitely until fertilized. At  $20^\circ\text{C}$ . this process has been completed in about 15 minutes. For at least 8 hours after this, the unfertilized eggs are uniform with respect to respiratory rate, and developmental potentialities.

### *Fertilizing the Eggs*

The eggs were fertilized in the manner described in the second paper of this series (19). It was found that spermatozoa in a shallow drop, even if concentrated, do not retain high fertilizing power very long when kept in the bulb of the vessel, in a  $\text{CO}_2$ -free atmosphere.<sup>1</sup> In most cases therefore the spermatozoa were either added directly to the opened vessel, after prefertilization measurements had been made on the eggs, or else placed in the bulb only about 10 minutes before insemination. In the former case no measurement was obtainable covering the first 10 or 15 minutes after fertilization, whereas in the latter case measurement was made covering the period beginning at insemination.

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<sup>1</sup> Compare Cohn (1) for possible explanation.

## RESULTS

Following Warburg (16) when KOH is used to absorb CO<sub>2</sub>:

$$x = h \left[ \frac{V_g \frac{273}{T} + V_f \alpha}{P_o} \right]$$

where  $x$  = mm.<sup>3</sup> O<sub>2</sub> consumed;  $h$  = change in pressure (observed) in millimeters manometer fluid (Brodie's fluid) at constant volume;  $V_g$  = volume in mm.<sup>3</sup> of the gas space from the sea water to the manometer meniscus;  $V_f$  = fluid volume in mm.<sup>3</sup>;  $\alpha$  = solubility coefficient of O<sub>2</sub> in sea water (Bunsen's coefficient);<sup>2</sup>  $P_o$  = standard pressure in millimeters of manometer fluid (10,000 mm. Brodie's fluid = 760 mm. Hg). The results are therefore expressed in mm.<sup>3</sup> O<sub>2</sub> at standard temperature and pressure, but corrections were not made for deviations from 760 mm. Hg of the actual barometric pressure in the laboratory.

Twenty-two separate measurements were made of the absolute rate of oxygen consumption by unfertilized eggs. The volume of eggs which had been used in a vessel was determined by centrifuging in a calibrated vaccine tube in which volume could be read to 1 mm.<sup>3</sup>. The eggs were centrifuged until no decrease in volume resulted from further centrifuging. Examination showed that the eggs were distorted enough to pack tightly, but there was no doubt some space between the eggs so that the volumes read (and used without correction) are slightly too great. Each value listed in Table II as from an independent measurement is the average of consecutive readings taken 5, 10, 15, 20, or 30 minutes apart for a total of between 40 and 65 minutes. The great majority are for 1 hour. The values are expressed in mm.<sup>3</sup> O<sub>2</sub> per hour per 10 mm.<sup>3</sup> eggs. The column (Table II) headed "additional measurements" represents values obtained from additional hourly measurements, after the 1st hour, upon the same eggs which are represented in the first column. The average value for the twenty-two independent measurements for the 1st

<sup>2</sup> The value of  $\alpha$ , the solubility coefficient of oxygen in sea water in parts per part, was calculated from Fox's (2) formula and tables, and from Page's (9) data on the salinity of Woods Hole sea water. At 21°C. the value of  $\alpha$  is 0.025. At 18°C. it is 0.027.

hour is 2.43 mm.<sup>3</sup> O<sub>2</sub> per hour per 10 mm.<sup>3</sup> eggs, with a probable error of  $\pm 0.13$ , while the average for the twenty-one additional measurements is 2.41. The very close agreement between these two groups is indicative of the reproducibility of the measurements on *Chaetopterus*

TABLE II

*The Rate of Oxygen Consumption by Unfertilized Chaetopterus Eggs*

Independent measurements	Mm. <sup>3</sup> O <sub>2</sub> per hour per 10 mm. <sup>3</sup> eggs	Additional measurements of approximately 1 hour each	Mm. <sup>3</sup> O <sub>2</sub> per hour per 10 mm. <sup>3</sup> eggs
1	2.0	1	2.5
2	2.5	2	2.5
3	2.5	3	1.9
4	2.0	4	2.4
5	2.4	5	2.2
6	2.6	6	2.2
7	2.6	7	2.8
8	2.7	8	2.5
9	2.5	9	2.5
10	2.6	10	2.5
11	2.5	11	2.5
12	2.4	12	2.5
13	2.6	13	2.7
14	2.5	14	2.4
15	2.1	15	2.1
16	2.5	16	2.5
17	2.4	17	2.4
18	2.2	18	2.4
19	2.5	19	2.4
20	2.4	20	2.4
21	2.4	21	2.3
22	2.5		
Average.....	2.43, P. E. 0.13	Average.....	2.41, P. E. 0.135

Over-all average (forty-three measurements) ..... 2.42, P. E. 0.13. temp. 21°C.  $\pm .05$

eggs, and further evidence of the lack of change in respiratory rate with longer shaking. The over-all average rate, forty-three cases, is  $2.42 \pm 0.13$  (P. E.)<sup>3</sup> mm.<sup>3</sup> O<sub>2</sub> per hour per 10 mm.<sup>3</sup> eggs.

<sup>3</sup> The computation of the probable error involves the assumption that the individual measures are scattered at random about the true value; i.e., that there is no consistent or directed error.



The change in rate of oxygen consumption after fertilization has been calculated as a percentage of the prefertilization rate of the same sample of eggs. The prefertilization rate was measured for from 40 minutes to  $1\frac{1}{2}$  hour before the eggs were inseminated. As is seen in Fig. 1, the rate during the 1st hour after fertilization rises only slightly. For convenience, the average rate for the 1st hour after fertilization, or that part of it measured, has been arbitrarily chosen for comparison with the prefertilization rate, and Table III shows the results of twenty-two such comparisons, with the rate of the fertilized eggs expressed as a percentage. The part of the 1st postfertilization hour measured is indicated, and also the percentage fertilization. In determining the percentage fertilization counts were made on at least 200 eggs. There is some room for error, however, especially in cases of low percentage fertilization, as it is at least possible that some eggs might be partially activated enough to undergo a respiratory change without giving morphological evidence of fertilization. I am led to suspect this in a few cases, such as the first experiment in Table III, where the respiratory change is greater than the observed percentage fertilization should bring about. The converse error does not exist. When 95 or 100 per cent of the eggs are unquestionably fertilized, the remainder represent so small a part of the whole, that it would make little difference if some of them were partially activated. It is much safer therefore to draw conclusions from Table III from those cases in which the percentage fertilization is high. It will be seen that the average for the 1st hour after fertilization is 53.3 per cent of the prefertilization rate in the six cases in which the percentage fertilization is 99 per cent or better. For the ten cases in which the percentage fertilization is 90 per cent or better, the average rate for the 1st hour is 54 per cent of the prefertilization rate. It seems safe to conclude that the true rate, assuming 100 per cent fertilization, is very close to 53 per cent. It is also seen that the eleven cases in which the percentage fertilization is less than 90 per cent, although they are of less value for absolute purposes, entirely bear out the results of the cases with high percentage fertilization. Without exception the rate of oxygen consumption drops following fertilization.

In ten experiments a measurement was made beginning immediately after insemination. The shortest interval so covered was 5 minutes.

In the first 5 or 10 minute period following insemination the eggs were found to respire at practically the same rate as in the second 5 or 10 minute interval, or at a very slightly lower rate. (See Experiment 5,

TABLE III

*Chaetopterus. The Rate of Oxygen Consumption by Fertilized Eggs Expressed as a Percentage of the Prefertilization Rate of the Same Sample of Eggs*

Experiment	Postfertilization time interval covered	Average rate, as percentage of prefertilized rate	Percentage fertilized
	<i>min.</i>	<i>per cent</i>	<i>per cent</i>
1	10-60	67	25
2	0-60	56	90+
3	10-30	50	90
4	0-60	66	75-
5	0-7	51	100
5	0-60	54	100
6	0-50	52	99+
7	0-60	51	99+
8	15-60	83	40
9	0-60	58	99
10	0-60	67	75-85
11	0-60	59	90+
12	0-60	54	90
13	0-60	51	99-
14	10-60	59	75
15	10-60	54	99
16	15-60	68	40+
17	15-60	70	40
18	10-60	55	95+
19	10-60	86	30-40
20	10-60	66	?
21	10-60	59	?
22	15-60	69	40-50
23	15-60	72	50

Average 6 experiments with 99-100 per cent fertilization 53.3 per cent

Average 5 experiments with 90-95 per cent fertilization 54.8 per cent

Average 11 experiments with 90-100 per cent fertilization 54.0 per cent

All other cases confirm drop in respiratory rate following fertilization.

Table III.) It therefore appears that the drop in rate at fertilization takes place very suddenly. With my apparatus it was not possible to make accurate readings at intervals more frequent than every 5

minutes. If there is any very extreme change at the instant of fertilization, comparable to that which Shearer (11) reports for the rise in rate in the sea urchin egg, it must be of such brief duration as to involve only a very small amount of oxygen. There is nothing in my results which indicates any such great change in rate.

At 21°C. the time after fertilization at which 50 per cent of a population of eggs have reached certain morphological stages was observed to be as follows:<sup>4</sup> first polar body, 14.5 minutes; second polar body, 27 minutes; pear-shaped stage, 46 minutes; polar lobe bulge, 52 minutes; cleavage with polar lobe attached, 58 minutes; complete cleavage with polar lobe resorbed into one blastomere, 62 minutes; 4 cell stage, 82 minutes. In order to discover if these events are accompanied by any change in respiratory rate it would be necessary to make measurements at very frequent time intervals<sup>5</sup> or else to adjust carefully the intervals of measurement for the purpose. This was not done in the present case, but enough measurements fall by chance at times to give some evidence, and to indicate that changes associated with these events are very slight if they exist at all.

Measurements were made most intensively and are most numerous covering the first hour and a half to 2 hours after fertilization. A number of measurements were made covering a longer period. 8 hours after fertilization, at 21°C., the *Chaetopterus* blastulae have developed cilia and are swimming actively. Four good measurements were made to about 8 hours, two more to about 7 hours, two more to about

<sup>4</sup> This schedule is for eggs which have been in sea water at least 15 minutes, to attain the resting stage in the metaphase of the first polar spindle. *Chaetopterus* eggs may be fertilized immediately upon removal from a parapodium, but in this case the development is delayed to the extent of the time required for the reorganization of the nucleus which also takes place in sea water in the absence of fertilization. At 25.5°C. samples of eggs were inseminated 30 seconds, 15 minutes, 30 minutes, 45 minutes, and 60 minutes after being placed in sea water. The time lapses from insemination until 50 per cent of the population cleaved was respectively: 59, 45, 46, 45, 46 minutes. In other words, eggs inseminated immediately upon being placed in sea water cleave only a minute earlier than eggs inseminated 15 minutes later. Eggs which have been in sea water an hour have the same time-lapse to cleavage as eggs which have been in sea water 15 minutes.

<sup>5</sup> J. Gray (3) has done this using the sea urchin egg and finds no change in the rate of oxygen consumption associated with cleavage.

6, and a larger number of various lesser lengths. These measurements, arranged as percentages of the prefertilization rate of the same sample of eggs in each case, and then set at the standard absolute rate, were all plotted on one graph. They include various time intervals between measurements. Fig. 2 shows the higher percentage fertiliza-

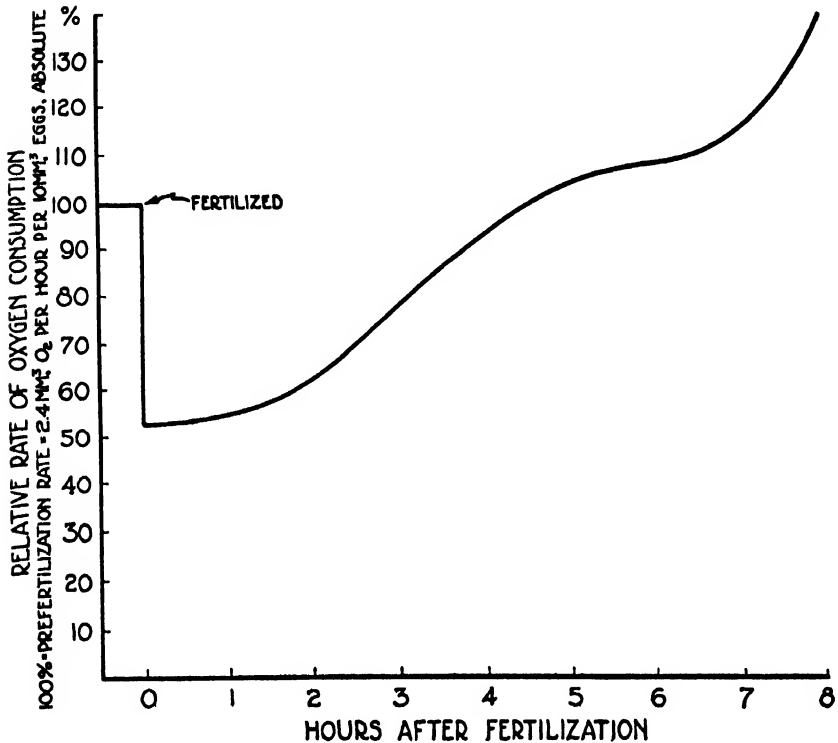


FIG. 1. The average relative rate of oxygen consumption by *Chaetopterus* eggs until 8 hours after fertilization, at 21°C. Ciliary activity develops as follows: 6 hours 15 minutes, a few embryos beginning to vibrate cilia slightly; 6 hours 30 minutes, a few very slowly rotating; 6 hours 45 minutes, about half swimming very slowly; 7 hours, most swimming fairly actively; 8 hours, very active, swimming fast and high.

tion data in this plot. A smooth resultant curve was drawn free hand and is given in Fig. 1. Fig. 2 thus gives the primary data on which the curve shown in Fig. 1 is based. Fig. 1 has also been influenced however by additional data, mostly from lower percentage fertilizations, and in the early part (first 2 hours) by a large number of other

measurements, results of which are given averaged for the 1st hour in Table III. These have been omitted from Fig. 2 because they are so superimposed that they could not be shown without great enlarge-

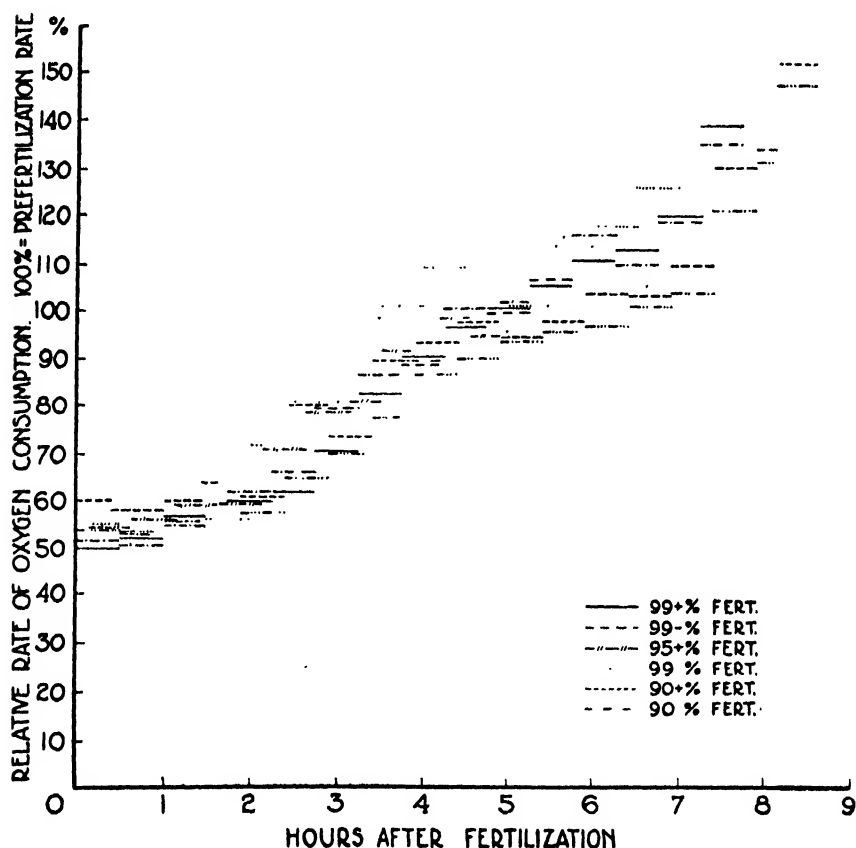


FIG. 2. The relative rate of oxygen consumption by fertilized *Chaetopterus* eggs at 21°C. These six series of actual measurements are the primary data upon which Fig. 1 is based (see text). Inspection of the individual series shows that after 5 hours the rise in respiratory rate with increasing ciliary activity is in each case much more like the curve in Fig. 1 than the whole grouping of measurements at first suggests. After 5 hours the several series have come to be slightly out of phase.

ment of Fig. 2. Until ciliary activity begins the curve is sigmoid between the time of fertilization and 6 hours or  $6\frac{1}{4}$  hours after fertilization. It is slightly asymmetric. The prefertilization rate is regained at between  $4\frac{1}{2}$  and 5 hours after fertilization. The development of

ciliary activity, with great increase in the rate of oxygen consumption, begins quite gradually and takes nearly 2 hours to get well under way. The times at which different degrees of ciliary activity have been attained are indicated in Fig. 1 (legend). This curve for the *Chaetopterus* egg is quite similar after fertilization to the corresponding curve for the sea urchin derived by J. Gray (4). The striking difference is in the opposite direction of the change at fertilization.

### *Arbacia punctulata*

After the drop in rate of oxygen consumption by *Chaetopterus* eggs following fertilization had been proven apparently beyond doubt, in the summer of 1930 I decided to make a few measurements of the change in the egg of the sea urchin *Arbacia*. An increase in rate of from 400 per cent to 700 per cent in the sea urchin egg has been so well established by different investigators, working on different species, that it seemed worth while to use this egg as a check on my apparatus and methods, even though I could not think of any possible gross error. For this purpose I made six measurements. Although the percentages fertilization were not especially good it was at once obvious that a great increase in rate of respiration followed fertilization. It was also found necessary to use much higher concentrations of unfertilized eggs as they respire at a much lower rate than the unfertilized *Chaetopterus* eggs. The results of these measurements are given in Table IV.

These data of 1930 (Table IV) are quite variable and therefore not altogether satisfactory. It is more difficult to make accurate measurements with these eggs than with *Chaetopterus* eggs because of the tendency of the *Arbacia* eggs, especially the unfertilized eggs, to cytolize when shaken. I did not find so clear-cut or definite a span of shaking rates over which the measured absolute rate was constant as in the case of the *Chaetopterus* eggs. A glance at Table IV will show that the measurements made with forty-eight round trip shakes per minute indicate a higher absolute rate than those with slower shaking. Are these too high due to cortical damage<sup>6</sup> or are the lesser rates too

<sup>6</sup> Cytolysis has repeatedly been found to greatly increase the rate of oxygen consumption by unfertilized sea urchin eggs. Hypertonic solutions or a variety of cytolytic agents have been found to have this effect by Warburg (14), Loeb

TABLE IV

The absolute rate of oxygen consumption by unfertilized *Arbacia* eggs and the relative rate in each case of the same sample of eggs after fertilization. The egg concentrations are expressed in terms of unit volume of eggs per units volume of sea water, and the shaker rate is round trip shakes per minute, with 7.5 cm. amplitude. Measurements made in 1930.

Experiment	Egg concentration	Rate of shaker	Oxygen consumption by unfertilized eggs in mm. <sup>3</sup> O <sub>2</sub> per hour per 10 mm. <sup>3</sup> eggs	Relative rate, eggs fertilized	Fertilization	Relative rate fertilized eggs, extrapolated to 100 per cent fertilization	Condition of eggs at end of measurements
				<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
1	1:11.5	48	0.47	215	50	320	Good
2	1:11	48	0.49	170	30	330	Small per cent of eggs slightly cytolized
3	1:17	36	0.30	233	15+?		10 per cent of eggs cytolized
4	1: 8	36	0.36	370	65	522	Excellent
5	1:10	36	0.42	209	25	560	Small per cent of eggs slightly cytolized
6	1:10	36	0.28	511	80	604	Good

low due to inadequate gas exchange? In favor of the former (assuming other workers' results to be approximately correct) is the relation between the absolute rate and the percentage increase at fertilization.

and Wasteneys (6), and Runnström (10). There is therefore great danger of obtaining too high a measurement of the rate of oxygen consumption by unfertilized eggs if damage is inflicted by too rapid shaking or in any other way. Both Wasteneys (17) (*Arbacia*) and Runnström (10) have found that unfertilized sea urchin eggs which are merely not entirely fresh; *i.e.*, after standing for some time in sea water, exhibit a decidedly elevated rate of oxygen consumption. Shearer (12) makes a point of recognizing the danger of increasing the rate of respiration and of heat production by cytolysis. My own observations on both *Arbacia* and *Fucus* eggs (unfertilized) are that respiratory increase is induced by too rapid shaking when the cytolysis is quite superficial and before it becomes visibly conspicuous.

The potential increase in respiratory rate of this unfertilized egg, so readily called forth by a wide variety of physical and chemical agents which tend to activate it, renders it poorly suited to some types of metabolism experiments which could more advantageously be performed with some other egg.

It is from an absolute rate for the unfertilized egg of about 0.4 (Table IV) that an increase of the order of fivefold follows fertilization. However these results are too meager to permit us to more than suppose the correct value to be of the order of 0.36 to 0.5 mm.<sup>3</sup> O<sub>2</sub> per hour per 10 mm.<sup>3</sup> eggs. (This compares with 2.4 for *Chaetopterus*.)

Due to relationships between the absolute rates of the different species of eggs which I found when organizing my data it became interesting to know the absolute rate, especially of the fertilized *Arbacia* egg, fairly accurately. In the summer of 1931 I decided, in hopes of greater accuracy, to confine some further measurements to the fertilized eggs, which had been fertilized in finger-bowls before being placed in manometer vessels. The higher rate of respiration of the fertilized eggs permits the use of less concentrated egg suspensions, and I hoped that the fertilization membrane would tend to protect the eggs from damage in shaking. There is also some reason<sup>7</sup> to suppose that the respiratory rate of the fertilized eggs, in which the great respiratory change at activation has already taken place, would be less affected by slight damage from shaking.

I used fresh sea urchins which I collected myself in Hadley Harbor in August. As in 1930 the gametes were obtained from the animals in the following way: With a pair of scissors the animals were cut in half around the equator. The upper half contained the gonads. The viscera and body fluids were removed and

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<sup>7</sup> Warburg (14) found that fertilized eggs treated with hypertonic solutions increased the rate of oxygen consumption after return to normal sea water by more than threefold. Loeb and Wasteneys (6) found that the rate of the unfertilized eggs was increased by such solutions to about the level of the fertilized eggs, but that the rate of fertilized eggs was not further increased. They found however (7, 8) (as Warburg had earlier) that the rate of oxygen consumption by fertilized sea urchin eggs is increased by bases especially when the egg cortex is "etched" or damaged, more so in *Arbacia* than in *S. purpuratus*. Runnström (10) finds that both hypertonic and hypotonic solutions, while increasing the rate of oxygen consumption by unfertilized eggs, decrease that of the fertilized eggs. Warburg (15) found that complete destruction of the fertilized egg cells results in a decrease in the rate of oxygen consumption. These discrepancies may be partly if not largely dependent upon different degrees of damage or destruction of the cells. It appears probable that a certain moderate degree of cortical damage might abnormally increase the rate of oxygen consumption by fertilized eggs as it unquestionably does in the case of the unfertilized sea urchin eggs.



the half shell was inverted in a finger-bowl containing shallow sea water. *Arbacia* which are ripe extrude gametes in large quantities through the gonopores into the finger-bowl when prepared in this way. Only ripe gametes are extruded, and they receive no rough treatment as they may when strained from ovarian tissue through coarse cloth. Eggs were washed several times and were then inseminated. Before they were put in manometer vessels supernumerary spermatozoa were largely removed by further washing. Volumes were determined by centrifuging the eggs at the termination of the measurement in calibrated vaccine tubes until further centrifuging caused no further decrease in volume. Due to the presence of the fertilization membranes, and to some space which no doubt occurred between eggs (which distort however and pack tightly) the observed volumes are no doubt slightly too high. In most cases the space between the egg and the fertilization membrane was obliterated in centrifuging so that the membrane fitted tightly to the egg. The contents of the space beneath the membrane was probably forced out through a rupture in the fertilization membrane. In three cases the fertilization membranes were mostly thrown off in the centrifuge and lay in a separate layer above the eggs.

The results of sixteen measurements are shown in Table V. These results are fairly uniform and give a better measure of the rate of oxygen consumption by recently fertilized *Arbacia* eggs than is obtainable from Table IV.

The average of all measurements up to (but not including) the measurement covering the 1st hour after fertilization is 2.0 mm.<sup>3</sup> O<sub>2</sub> per hour per 10 mm.<sup>3</sup> eggs. This includes some measurements on eggs less than 100 per cent fertilized and is therefore on this score too low, but this is offset since the consecutive measurements shown in Table V show that the rate is rising with time even within the 1st hour after fertilization (apparently more rapidly than in *Chaetopterus*, see Fig. 1). The average of the first measured intervals of the nine series of measurements in which 98–100 per cent of the eggs were fertilized is negligibly below 2.0. The average time interval after fertilization of these measurements is the period 23–38 minutes, with limits of 20 and 45 minutes after fertilization. The rate at a time still closer to fertilization would probably be slightly lower (omitting the brief great rise which Shearer (11) reports in the 1st minute after fertilization in the sea urchin *Echinus microtuberculatus*).

From the data in Table IV on unfertilized eggs, and in Table V on fertilized eggs, the percentage increase following fertilization can be estimated, being limited in accuracy by the measurements on the

TABLE V

Fertilized *Arbacia* eggs. All measured periods up to 1 hour after fertilization, including lesser percentages fertilization, averaged, rate = 2.0 mm.<sup>3</sup> O<sub>2</sub> per hour per 10 mm.<sup>3</sup> eggs. Nine cases with 98 to 100 per cent fertilization, first measured periods averaged (average time interval = 23-38 minutes after fertilization) = 2.0 mm.<sup>3</sup> O<sub>2</sub> per hour per 10 mm.<sup>3</sup> eggs.

Showing that respiratory rate is rising: average of all 15 minute measurements (of runs which go at least to 80 minutes after fertilization) terminating at or including the time: 50 minutes after fertilization, (twelve cases not all 100 per cent fertilization) 2.2. Same twelve cases at 80 minutes: 2.7; seven cases at 95 minutes: 3.1.

Ex- peri- ment	Egg suspension	Mm. <sup>3</sup> eggs	Ratio egg volume: sea water volume	Shaking rate round trip shakes per min. 7.5 cm. amplitude	Fertili- zation	Period after fertili- zation of measure- ment	Absolute rate mm. <sup>3</sup> O <sub>2</sub> per hour per 10 mm. <sup>3</sup> eggs	Condition of eggs at end of experiment
	cc.				per cent	min.		
1	4	17	1:235	40	100	30-45 45-60 60-75	2.2 2.8 2.1	Excellent. Less than $\frac{1}{2}$ per cent show slight cy- tolysis.
2	4	84	1:47	40	80	25-40 40-55 55-70	1.2 1.5 1.5	Excellent. No cytolysis.
3	4	83	1:48	40	80	25-40 40-55 55-70	1.5 1.8 1.8	" "
4	2	35	1:56	40	75	25-40 40-55 55-70	1.0 2.7 1.5	Excellent. No cytolysis.
5	4	141	1:27	40	100	25-40 40-55 55-70 70-85	2.5 2.9 2.7 2.8	Excellent. Perfect cleavages.  No cytolysis.
6	4	139	1:28	40	100	25-40 40-55 55-70 70-85	2.4 2.7 2.5 2.7	" "

TABLE V—*Continued*

Ex- peri- ment	Egg suspension	Mm. <sup>3</sup> eggs	Ratio egg volume: sea water volume	Shaking rate round trip shakes per min. 7.5 cm. amplitude	Fertili- zation	Period after fertili- zation of measure- ment	Absolute rate mm. <sup>3</sup> O <sub>2</sub> per hour per 10 mm. <sup>3</sup> eggs	Condition of eggs at end of experiment
	cc.				per cent	min.		
7	4	74	1:55	40	95	25-40 40-55 55-70 70-85	2.3 2.7 2.8 3.6	No cytolysis.
8	4	63	1:63	40	100	20-35 35-50 50-65 65-80 80-95	1.3 1.8 2.6 2.3 2.7	" "
9	4	62	1:63	40	99	20-35 35-50 50-65 65-80 80-95	1.4 1.8 3.0 2.3 3.0	Excellent. Perfect cleavages.  No cytolysis.
10	4	63	1:63	40	99	20-35 35-50 50-65 65-80 80-95	2.0 2.2 3.2 3.0 3.0	" "
11	4	90	1:43	40	85	37-52 52-67 67-82	1.4 1.5 1.8	Good. No cytoly- sis.
12	4	89	1:43	40	85	37-52 52-67 67-82	1.5 1.7 1.8	Good. No cytoly- sis.
13	2	100	1:19	50	96	25-40 40-55 55-70 70-85 85-100 100-115 115-130	1.5 1.9 2.4 2.7 3.1 3.0 3.0	Excellent. Trace of cytolysis affecting less than 5 per cent of eggs.

TABLE V—*Concluded*

Ex- peri- ment	Egg suspen- sion	Mm. <sup>3</sup> eggs	Ratio egg volume: sea water volume	Shaking rate round trip shakes per min. 7.5 cm. amplitude	Fertili- zation	Period after fertili- zation of measure- ment	Absolute rate mm. <sup>3</sup> O <sub>2</sub> per hour per 10 mm. <sup>3</sup> eggs	Condition of eggs at end of experiment
	cc.				per cent	min.		
14	2	96	1:20	50	98	25-40 40-55 55-70 70-85 85-100 100-115 115-130	2.1 2.1 2.6 3.0 3.4 3.2 3.3	Excellent. No cytolysis. Perfect cleavages.
15	2	46	1:42	50	100	23-38 38-53 53-68 68-83 83-98	1.2 2.5 2.5 2.8 3.1	Excellent. Perfect cleavages.  No cytolysis.
16	2	45	1:43	50	100	23-38 38-53 53-68 68-83 83-98	2.7 3.0 2.8 3.3 3.1	Excellent. Perfect cleavages.  No cytolysis.

unfertilized eggs. If the value 0.4 is accepted for the unfertilized eggs the increase is approximately fivefold. If the higher value 0.5 is taken the increase is about fourfold. Loeb and Wasteneys (5) found an increase of nearly or about fourfold for this species. Tang (13) reports an increase of fivefold. In applying my data on *Arbacia* in Fig. 1 (21) I am using the value 0.4-0.5 mm.<sup>3</sup> O<sub>2</sub> per hour per 10 mm.<sup>3</sup> eggs for the unfertilized eggs, and 2.0 for the fertilized eggs. These absolute values are very considerably below Tang's absolute values. This discrepancy will be considered in the fifth paper of this series (21).

## SUMMARY

1. Unfertilized eggs of *Chaetopterus* consume about 2.4 mm.<sup>3</sup> O<sub>2</sub> per hour per 10 mm.<sup>3</sup> eggs at 21°C.
2. In the 1st hour after fertilization, the fertilized eggs consume

oxygen at about 53 or 54 per cent of this rate, which is about 1.3 mm.<sup>3</sup> O<sub>2</sub> per hour per 10 mm.<sup>3</sup> eggs at 21°C.

3. For the first 6 hours after fertilization, at 21°C., the curve of the rate of oxygen consumption is slightly asymmetrically sigmoid. The prefertilization rate is regained between 4½ and 5 hours after fertilization. Soon after 6 hours, ciliary activity begins, and the rate of oxygen consumption rises rapidly.

4. The unfertilized eggs of *Arbacia punctulata* consume about 0.36–0.5 mm.<sup>3</sup> O<sub>2</sub> per hour per 10 mm.<sup>3</sup> eggs at 21°C. The absolute determination is difficult as these eggs are highly sensitive to shaking in the manometer vessels, and these difficulties are discussed.

5. The fertilized eggs of *Arbacia punctulata* consume oxygen at the rate of about 2.0 mm.<sup>3</sup> O<sub>2</sub> per hour per 10 mm.<sup>3</sup> 21°C. At 1 hour after fertilization the rate is already rising.

6. A comparison of the absolute rates of oxygen consumption, and the changes in rate at fertilization of these and a number of other eggs, together with a theoretical discussion, and a discussion of discrepancies in measurements on the eggs of *Arbacia punctulata*, is contained in the fifth paper of this series (21).

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# ON THE RATE OF OXYGEN CONSUMPTION BY FERTILIZED AND UNFERTILIZED EGGS

## V. COMPARISONS AND INTERPRETATION

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In 1895 Loeb (25) found that the segmentation of fertilized sea urchin eggs is inhibited when oxygen is displaced from the medium with hydrogen. He later found the same effect on a variety of eggs, and further that suppression of oxygen consumption by KCN caused the same result. If the suppression of oxidation by either method was not too prolonged the inhibition was reversible. When immature starfish eggs are placed in sea water maturation takes place, before fertilization, and Loeb (28) found that this nuclear activity was also reversibly inhibited either by oxygen lack or by KCN. These facts led to the conclusion that oxidation, or more strictly oxygen utilization, is necessary for the following cell activities: any protracted nuclear activity, cell division, and development. Oxidation and these activities go together. Loeb (26, 29) proposed that the essential feature or one of the essential features of fertilization, the initiation of these activities, is an increase in the rate of oxidations of the resting egg cell.<sup>1</sup> A large body of evidence from the sea urchin egg, some of which will be considered presently, supported this view and led to its elaboration. The point which I wish to emphasize is that this involves the supposition

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<sup>1</sup> Loeb in 1916 (29, page 117) says "These conclusions have been since amply confirmed by the measurements of O. Warburg as well as those of Loeb and Wasteneys, both showing that the entrance of the spermatozoön into the egg raises the rate of oxidation from 400 to 600 per cent. . . ."



that the inhibition of these activities in the unfertilized egg is due to low oxygen utilization (oxidative<sup>2</sup>) rate as a limiting factor (as it evidently is in fertilized eggs deprived of oxygen). Loeb was perhaps right in supposing this for the sea urchin egg. At least by implication (29) he applied this explanation to fertilization in general, and it is the generalization which I believe to be entirely refuted by my data from *Chaetopterus* (60) and *Cumingia* (58), in which the rate of oxygen consumption drops sharply at fertilization. The generalization has already been seriously questioned (20, 13) on the basis of measurements on other eggs which show little if any increase following fertilization. Further, the general relations between oxidation rate and development, and oxidation and anesthesia (inhibition) do not favor increased oxidation as in general a causal factor in the initiation of development.

In 1906 Loeb (27) found that either removal of oxygen or addition of KCN prevented the rapid disintegration of sea urchin eggs which follows a number of treatments which induce artificial membrane formation. This suggested a connection between the disintegration of the cortex, which also occurs to a lesser extent in the normal fertilization of a number of eggs including the sea urchin, and increased oxidation rate.<sup>3</sup> He found further that the fertilized sea urchin eggs produce more acid than the unfertilized eggs. These relations, in conjunction with the suppression of development by oxygen lack, led him to suppose that the rate of oxidations in the egg is increased when the egg is activated, and he expressed the view (26) already quoted that this is the essential feature or one of the essential features of fertilization. In the decade to follow, Loeb and Loeb and Wasteneys published an extended series of papers giving the results of many experiments especially with parthenogenetic agents and the effects of these upon the oxidative metabolism. Warburg (50) was the first to prove directly that the oxygen-consumption rate of the sea urchin egg increases greatly at fertilization. By the Winkler method he found at Naples that the increase in *Arbacia pustulosa*, within the 1st hour

<sup>2</sup> Loeb uses the general term "oxidation" in these connections when using data which measure oxygen consumption. See also paragraph 14 in the Summary.

<sup>3</sup> It is important however that such an anesthetic as chloral hydrate has the same effect without appreciably reducing the oxidation rate.

after fertilization is six- or sevenfold. Loeb and Wasteneys (30) later found an increase of nearly fourfold in the American *Arbacia punctulata*, and an increase of four- to sixfold in the California sea urchin, *Strongylocentrotus purpuratus* (33). Increases of the same order of magnitude have been measured in *Echinus miliaris* by Shearer (46) and by Gray (13), in *E. microtuberculatus* by Shearer (45), in *Paracentrotus lividus* by Warburg (55) and by Runnström (44), and recently again in the *Arbacia punctulata* by Tang (47) and by myself (60).

Warburg, and Loeb and Wasteneys, found that hypertonic solutions increase the rate of oxygen consumption by unfertilized sea urchin eggs toward or to the rate of fertilized eggs. Runnström (44) has shown that hypotonic solutions as well have this effect. In short, it was shown by Warburg and, with certain disagreements, confirmed by Loeb and Wasteneys, that hypertonic solutions and bases increase the rate of oxygen consumption by unfertilized eggs. Warburg (41) also showed that this is true of certain metals which act as parthenogenetic agents. However, to conclude that activation of the egg by these agents, mostly cytolytic, is brought about because they have increased the rate of oxidation involves some danger of reasoning in a circle so long as the oxidation-increasing effect of these agents is determined only on the unfertilized sea urchin egg. It does not follow except by arbitrary assumption which is cause and which is effect. Perhaps these agents increase the rate of oxidation only because they have (otherwise)<sup>4</sup> activated the egg. The effect of the cytolytic agents on the rate of oxygen consumption by fertilized eggs or other organisms gives evidence which is free from this objection. Warburg (51) did find that hypertonic solutions increase the rate of oxygen consumption by fertilized eggs some threefold, but Loeb and Wasteneys (33) found that in *S. purpuratus* hypertonic solutions increase the rate of oxidation only in unfertilized eggs, not in fertilized eggs. They concluded that the oxidation is increased only when formation of the fertilization membrane is brought about.<sup>5</sup> Complete cytolysis of the

<sup>4</sup> And in a way which may effectively activate other species of eggs without causing an increase in the rate of oxygen consumption.

<sup>5</sup> It was later necessary to modify this to whenever the condition is brought about which normally gives rise to the fertilization membrane.

unfertilized eggs by saponin increased the rate of oxygen consumption to the level of fertilized eggs, but no further. Warburg (54) also found that complete destruction of the fertilized egg cells caused a drop in the rate of oxygen consumption toward the prefertilization rate, and Runnström (44) finds this effect by both hypertonic and hypotonic solutions on the fertilized eggs of *P. lividus*. There is thus some disagreement as to the effect of cytolysis on the fertilized eggs, possibly due in part to different degrees of destruction.

Bases also act as parthenogenetic agents. Warburg (52) found that strong bases increase the rate of oxygen consumption of fertilized as well as of unfertilized eggs. Loeb and Wasteneys (35) found that both weak penetrating and strong bases increase the rate of oxygen consumption by both unfertilized and fertilized eggs. More recent work on many organisms has established that bases do tend in general to increase the rate of cell oxidation, quite apart from the activation of eggs, and at least in some cases cytolysis does also<sup>6</sup> (Shearer (46)). It is reasonable to suppose that agents which tend in general to increase respiratory rate may be acting parthenogenetically because of this effect. However, fatty acids and fat solvent anesthetics which act as parthenogenetic agents tend in general to suppress oxidation. It is true that anesthetics may increase the rate of oxygen consumption by the unfertilized eggs (having activated them) but to a lower level than is achieved by fertilization. Anesthetics decrease the rate of respiration of fertilized eggs. In other words they activate unfertilized sea urchin eggs and at the same time suppress the new rate of oxygen consumption. Loeb (41) found that the presence of oxygen is not necessary for the parthenogenetic action of fatty acids<sup>7</sup> and fat-solvent anesthetics. Lyon (37) found that prolonged exposure to KCN activates the eggs of a Mediterranean sea urchin. Therefore even for the sea urchin the case is not so very strongly in favor of the oxidation-increasing properties of parthenogenetic agents, acting directly as such, as the principal causal factor in the activation of the egg.

<sup>6</sup> It would be of value to know if cytolysis as such increases the rate of oxygen consumption by the unfertilized *Chaetopterus* egg since activation of this egg results in a decrease (60).

<sup>7</sup> See also Ralph Lillie (24) on aerobic and anaerobic phases of activation in the starfish egg.

Loeb's hypothesis as outlined in his book, *Artificial parthenogenesis and fertilization*, 1913 (28), and more briefly in his last somewhat extended review of the subject in 1916 in his book, *The organism as a whole* (29), may be briefly abstracted as follows: Parthenogenetic agents are in general cytolytic agents. Their effect is achieved by a superficial cytolysis of the egg cortex. Cytolysis as such, and perhaps especially superficial cytolysis of the cortex, increases the rate of cell oxidation. This increase in the rate of oxidation in parthenogenesis as in normal fertilization is the essential or one of the essential features of fertilization (activation), increased oxidation rate permitting or causing the developmental activities. Probably the important feature of the cortical cytolysis is the alteration or destruction of a lipoid film, and the increase in rate of oxygen consumption which follows may be due, for example, to the liberation thereby of some oxidative catalyst<sup>8</sup> from the film. This hypothesis of Loeb's is supported in some of its steps by a wealth of experimental data, concisely reviewed in 1916 (29), and accounts well for some of the extensive and diverse results of experiments on parthenogenesis of the sea urchin egg. But even for parthenogenesis in this egg, the case does not appear to be entirely conclusive, for reasons just set forth.

Loeb and Wasteneys (32) were the first to measure the rate of oxygen consumption before and after fertilization by any egg other than that of the sea urchin. In five measurements on eggs of starfish, two of which had more than 50 per cent fertilization, they found very little change and concluded that there is no change at fertilization in this egg. Since the essential feature of fertilization is entirely present here, *i.e.* the transformation of the egg from a comparatively resting condition to a state in which growth and differentiation are initiated, this appears at once to prevent generalization of Loeb's interpretation devised for the sea urchin egg as Frank Lillie (20) points out. Loeb and Wasteneys anticipated this objection at the time of their measurements on the starfish egg however by pointing out that the starfish egg is already active in maturation before fertilization and is there-

<sup>8</sup> This tentatively proposed mechanism for the increase in rate of oxygen consumption is not a necessary part of the hypothesis. The liberated catalyst could as well be in the cell interior. See Runnström (44) for an analysis of the mechanism of increase.

fore (supposedly) already at the high rate before fertilization. But even though there is nuclear activity before fertilization in this egg, there is still a very radical further increase in activity of the egg at fertilization, with no accompanying increase of respiratory rate, so it is difficult to bring this egg into a generalization of Loeb's hypothesis. Further, since no absolute measurements were made in terms of volume, weight, or number of cells no more proof was actually presented to show that the rate of the starfish egg is high throughout than that it is low, although they were probably right in supposing it to be high compared with the unfertilized sea urchin egg. Recently Tang (48) has made a few measurements on the same egg (*Asterias*). He also finds no appreciable change at fertilization. He gives his results in absolute units in terms of the number of eggs ( $160\mu$  in diameter). When converted to volume units,  $\text{mm.}^3 \text{ O}_2$  per hour per  $10 \text{ mm.}^3$  eggs, at  $23^\circ\text{C}$ . the value becomes about 0.8 which (compare Fig. 2) is relatively low but still about twice as high as Shearer's, Warburg's, and my own measurements on the unfertilized eggs of as many species of sea urchins. Tang's measurements were made with a rapid rate of manometer shaking which would seriously damage most kinds of eggs. He finds that when compared with his own values [which are considerably higher than mine (60)] for *Arbacia* eggs, the two unfertilized eggs consume oxygen at about the same rate per unit surface. The fertilized eggs therefore of course do not. On this basis of comparison his results directly oppose Loeb and Wasteney's assumption that the starfish egg operates at a high rate before fertilization.

Measurements of the rate of oxygen consumption by the eggs of other invertebrates have in no instance revealed such a rise following fertilization as that which occurs in the sea urchin egg. Fauré-Fremiet (9) found only a slight increase in the eggs of the polychaete *Sabellaria alveolata*, of the order of 12 per cent at  $20^\circ\text{C}$ . My own measurements using the eggs of the brown alga *Fucus vesiculosus* (57) show an increase of about 90 per cent, and in *Cumingia* (58) and *Chaetopterus* a drop following fertilization. I found the egg of *Nereis* (59) to increase its rate of oxygen consumption about 35-45 per cent following fertilization.<sup>9</sup>

<sup>9</sup> Barron and Tyler measured the change in this egg before I did. Barron (3) concludes that there is an increase of about 25 per cent immediately after fertiliza-

Among the eggs of vertebrates there is also no uniform behavior. Several investigators have found an increase following fertilization in amphibian eggs (see Needham's text (40)). Gray (13) says, "A sudden increase of respiration following fertilization is not shown by other eggs than those of the sea urchin," and in 1919 Lillie (20, pages 145-146) in effect predicts that eggs may be found which like the starfish do not change much, or which may even decrease, as has turned out to be the case for *Chaetopterus* (60) and *Cumingia* (58).

The question then arises, does the change in rate of oxygen consumption at fertilization, when present, have any direct relation to fertilization as such, or is it as chaotic a relationship as the divergent results might suggest? In the first place so many eggs change abruptly in one direction or another at exactly this time, that it seems highly probable that there is some definite relationship. On general physiological grounds it is to be expected that if efficiency remains constant an increased rate of oxygen utilization is required in aerobic systems if growth and development are speeded up, but it is well established that the rate of development is not ordinarily limited by the rate of oxidation. Cleavage rate is certainly not ordinarily limited by the rate of oxygen consumption in a number of forms, e.g. Amberson (2) found that the rate of cleavage in *Arbacia* eggs is not retarded, when the oxygen pressure is diminished, until the pressure has been so greatly reduced as to decrease the rate of oxygen consumption to about half normal. Loeb and Wasteneys (31) showed that the temperature coefficient of cleavage in *Arbacia* is entirely different from

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tion, but that about 18 minutes after fertilization the rate declines to the prefertilization rate. Barron's results, as far as they go agree entirely with my own. But following fertilization the *Nereis* egg secretes a large amount of jelly which with the sea water makes a solid gelatinous mass unless the eggs are very dilute. This gelation suppresses the observed rate of oxygen consumption. I found (59) that the magnitude of the decline beginning about 18 minutes after fertilization depends upon the concentration of eggs and the degree of gelation of the medium. In high concentration the drop is to well below the prefertilization rate, while when the eggs are so dilute that the medium is fluid, the drop is by no means down to the prefertilization rate. Barron suggests that the depression beginning 18 minutes after fertilization may be due to CO<sub>2</sub> retention by the jelly. It appears therefore that the increased rate immediately following fertilization in this egg is at least largely maintained if excessive concentration of the eggs is avoided.

the temperature coefficient of oxygen-consumption rate. It is quite possible that the rate of oxygen consumption is a direct part of the limiting factor which inhibits the unfertilized eggs of some species, and not of others. An egg so inhibited might be expected to have a relatively low rate of oxygen consumption before fertilization, as does the sea urchin egg. Runnström (44) concludes from experiments in which he analyzes by steps the ability of both the unfertilized and the fertilized sea urchin egg to carry out the steps in the oxidation process, that the oxygen-activating ferment or "*Atmungsferment*" is not fully in contact with its substrate in the unfertilized egg, but becomes so at fertilization, in conjunction with colloidal changes in the protoplasm. In this case the inhibition to development may be directly due to the oxidation limitations. This situation in the sea urchin egg would agree essentially with Loeb's hypothesis, while supplying a more modern and exact mechanism for the increase in rate of oxygen consumption at fertilization. The results of similar experiments on the eggs of *Chaetopterus* would be interesting.

It is more difficult to imagine the relation between the high rates of oxygen consumption by the unfertilized eggs of *Chaetopterus* and *Cumingia* and their state of inhibition. Both of these, like the starfish egg, undergo maturation activities in the sea water before fertilization, but only as far as the metaphase of the first polar spindle. In *Chaetopterus* this activity is over in 15 minutes at 21°C., and thereafter the egg is morphologically at rest. Even so, the high metabolic rate continues uniformly for at least 8 hours. Evidently, some condition other than limitations imposed by the rate of oxygen utilization must be responsible for inhibiting the development of the egg, since when it is fertilized and developmental activity is initiated, it proceeds with a much reduced rate of oxygen consumption.

When the absolute rates of oxygen consumption by the several species of eggs, in mm.<sup>3</sup> O<sub>2</sub> per hour per 10 mm.<sup>3</sup> eggs at 21°C., are compared it is found that the rates are much closer to one another after fertilization than before. *Chaetopterus*, *Cumingia*, and *Nereis* are strikingly close, while *Arbacia* is somewhat higher. Thus the range of these four eggs before fertilization is 0.4 or 0.5 to 3.1, a range of more than sixfold, while after fertilization it is only 1.3 to 2.0 (Fig. 1). Comparison with certain other growing animal cells of roughly com-

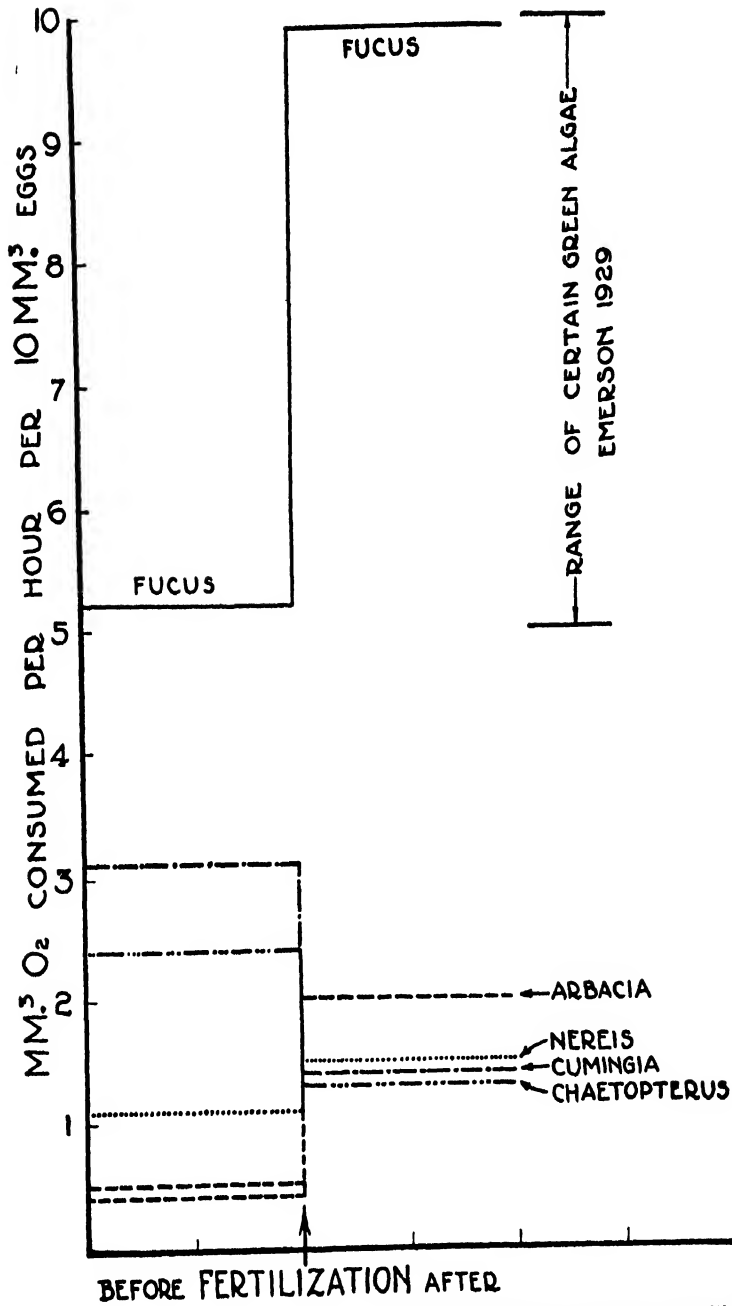
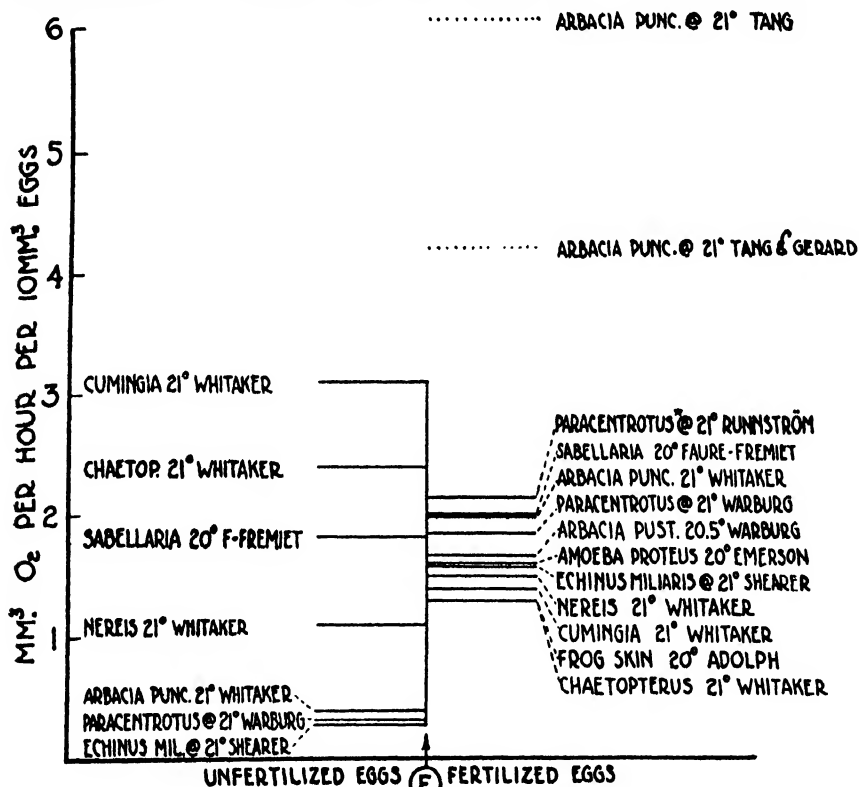


FIG. 1. Absolute rates of oxygen consumption, before and after fertilization at 21°C., except *Fucus* which is at 18°C.



comparable size, and which are not actively motile, shows a number of comparable values, e.g. Emerson's (8) measurement on *Amoeba proteus* at 20°C., for which the quotient is 1.6. I have also converted as many measurements on the eggs of marine invertebrates as I have been able to find in the literature together with sufficient data to make the conversions, into volume units for comparison. Since a variety of



\*WOULD PRESUMABLY BE LOWER IF MEASUREMENTS WERE CONFINED TO FIRST HOUR AFTER FERTILIZATION; SEE APPENDIX.

FIG. 2. Absolute rates of oxygen consumption, per unit volume, compiled. Where temperature is preceded by the symbol @, a temperature correction has been made. (See appendix.)

units have been used by different workers in some cases assumptions, e.g. of density, have been necessary for these approximate conversions. For temperature corrections the coefficient  $Q_{10} = 2$  has been assumed. The assumptions and the sources of data are given in the appendix. The compilation, given graphically in Fig. 2 shows that a number of fertilized eggs of several phyla fall approximately within this same

range, 1.3–2.0 mm.<sup>3</sup> O<sub>2</sub> per hour per 10 mm.<sup>3</sup> eggs at 21°C. The values for the *Arbacia punctulata* of Tang (47) and Tang and Gerard (49), unlike my own (60) are outside this general range. The relative similarity of the rates of oxygen consumption per unit volume by these various fertilized eggs, some of which increase to a greater or lesser extent, and some of which decrease the rate of oxygen consumption following fertilization suggest a certain orderly basis for the directions and magnitudes of the changes. It is as if the change at fertilization is in the direction, and of such magnitude, as to bring the rate of the fertilized eggs to the approximate rate 1.3–2.0 mm.<sup>3</sup> O<sub>2</sub> per hour per 10 mm.<sup>3</sup> eggs at 21°C. Eggs which have a very low prefertilization rate (notably the sea urchins) increase greatly, while those with a very high rate (*Chaetopterus* and *Cumingia*) decrease. Those in the middle range (*Nereis* and *Sabellaria*) change little.<sup>10</sup>

The wide differences in rate of oxygen consumption by the various unfertilized eggs is still unaccounted for. It is not difficult to asso-

<sup>10</sup> It remains to be seen how widespread this relation will be, but too many cases fit in for it to be purely a chance assortment. It is by no means to be expected that all or even most fertilized eggs will fit closely into this narrow range on a volume basis. Not only are there probably different characteristic concentrations in different species of eggs of the substances which determine the rate of oxygen consumption, but different proportions of inert materials occur for which it would be difficult to make accurate allowance. Especially, larger eggs which contain proportionally more yolk must be expected to respire at a lesser rate per unit volume, and this is the case for the absolute rates of the eggs of the frog (5) the Plaice egg (7) and for *Fundulus* (6). My own measurements on the eggs of the brown alga *Fucus* (57) indicate a rate entirely too high to fit into this system. This is probably in part due to the relatively small volume of water and inert storage in the *Fucus* eggs, which although very small settle so much more rapidly in sea water than animal eggs as to indicate a considerably greater density. When the *Fucus* egg is centrifuged, the inclusions come to occupy a relatively small part of the volume of the egg. Fig. 1 shows that the *Fucus* egg has a respiratory rate of the same order of magnitude as several small-celled algae (8) which appear in general to respire at a higher rate than immotile animal cells. The eight eggs and the *Amoeba* in Fig. 2 are all small cells which probably have a roughly comparable volume of active protoplasm per unit volume of cells. Tang's (48) absolute value for the eggs of the starfish *Asterias* when converted to volume units indicates a comparatively low rate for this fairly large and comparatively yolky egg (160μ in diameter). Both before and after fertilization it is about 0.8 mm.<sup>3</sup> O<sub>2</sub> per hour per 10 mm.<sup>3</sup> eggs at 23°C.

ciate the low rate of the unfertilized sea urchin egg with the inhibited resting condition of the cell. It is more surprising<sup>11</sup> to find the high rates of *Cumingia* and *Chaetopterus* when the eggs are in the inhibited resting state. It should be emphasized that the inhibited unfertilized egg is a cell in a very unusual condition. It is young; it contains all of the substances necessary for rapid growth, yet it halts suddenly in a state of suspended animation until its condition is in some way changed by any one of a variety of external stimulating agents. As soon as activation has taken place it again behaves as an ordinary cell, with division and growth, like a protozoon or somatic cell when conditions are favorable. It is after the return to this ordinary state that the rate of oxygen consumption by the different eggs are similar, and similar also to the rates of *Amoeba* and the frog's skin. The relations seem to me to suggest that the diverse rates of oxygen consumption, like the cell division and growth phenomena, are in an extraordinary state before fertilization, being related thereto in a manner which is in most cases at present obscure, and that fertilization is a termination of this unusual condition. This is not wholly a new idea. Fertilization has often been regarded as the termination of an inhibition. I wish to point out that these oxygen consumption relations conform to this point of view. The eventual generalized interpretation of fertilization may well resolve itself into an analysis of the nature of the inhibition of the unfertilized egg.

### *The Inhibition of the Unfertilized Egg*

The behavior of the unfertilized egg suggests certain resemblances between its condition and that of a cell under the influence of an anesthetic. The resemblance may be partly superficial but it is probably not entirely so. It is of course not to be supposed that the unfertilized egg is actually subjected to an anesthetic substance from without. Rather it may be that the unfertilized egg cell, upon reaching its "resting stage," has spontaneously assumed the condition, whether it be related to the structure and composition of the surface

<sup>11</sup> Surprising in so far as we expect the organism to be efficient. It is not justifiable, however, either philosophically or biologically, to suppose that the organism will necessarily adjust its fuel consumption to a low level when apparent useful work is at a minimum.

film, or to the degree of dispersion and the nature of the adsorptions on the plasma colloids, similar to that which anesthetics impose on cells. The failure of cutting to activate eggs may favor the supposition that the condition resides in the plasma rather than exclusively in the cell surface film, although disruption of the lipoid surface film is supposed by Loeb (29) and by Gray (11) (with different explanations of how it is brought about in normal fertilization) to result in activation of the egg.

If the inhibition or autoanesthesia of the unfertilized sea urchin egg is caused by the very low rate of oxygen consumption as a limiting factor, this inhibition is then an "asphyxiation anesthesia." While as has been stated, this explanation is tenable for the sea urchin egg, it is not to be expected from the general relations between anesthesia and oxidation that inhibitions will generally be "asphyxiation-anesthesia." Loeb and Wasteneys (34), among others, have shown that while anesthesia, caused by narcotics, tends to suppress the rate of oxygen consumption it does not do so at all sufficiently for this to be the limiting factor. Lowering the temperature a few degrees will lessen the oxygen consumption to the same extent while by no means causing anesthesia. They conclude that anesthesia is not ordinarily an "asphyxiation" phenomenon. Loeb and Wasteneys (31) have also shown that the rate of development of the fertilized sea urchin egg is not directly dependent upon the rate of oxygen consumption; *i.e.*, that "oxidation is not the independent variable" in development. They showed that the temperature coefficient of development is variable over a temperature range and decidedly not the same as the temperature coefficient ( $Q_{10}$  = approximately 2) of oxygen consumption, as it would be if development were a direct dependent variable. It is thus quite in conformity with these relationships to find eggs which do not have a low rate of oxygen consumption, responsible for the inhibition, in the unfertilized condition. Examples are known in which mild anesthesia is actually accompanied by an increased rate of respiration (38, 42).

Heilbrunn (15) has shown that the apparent viscosity or consistency of the protoplasm of the eggs of both *Arbacia* and *Cumingia* changes greatly at or soon after fertilization so that the degree of gelation is increased. Before fertilization, in the inhibited condition, the proto-

plasm is comparatively fluid.<sup>12</sup> Anesthetics cause the protoplasm of these and other cells to liquefy. If the fertilized egg is inhibited by means of an anesthetic it becomes less gelated, as it was before fertilization. Heilbrunn believes that the colloidal condition of the protoplasm, as revealed by the consistency, is causally related to anesthesia, or on the other hand to stimulation and increased activity. It is notable here that release from inhibition (fertilization) in *Arbacia* and in *Cumingia* are accompanied by the same types of changes in consistency (correlating with liberation from anesthesia), while the changes in rate of oxygen consumption run in the opposite direction and must therefore be eliminated from the correlation.

Ralph Lillie (21, 22, 23) has shown that the permeability to water of the *Arbacia* egg increases following fertilization or activation, and he draws parallels on this basis between activation of the egg and stimulation in general. In *Arbacia* the increase in water permeability is of about the same order of magnitude as the increase in oxygen consumption rate,<sup>13</sup> while in the starfish egg (23), in which the rate of oxygen consumption does not change at fertilization, neither does the permeability to water change at fertilization. It is therefore interesting to note that the correlation between permeability and rate of respiration breaks down when the *Cumingia* egg is considered, as Heilbrunn (14) has found that the permeability to water of the *Cumingia* egg increases slightly following fertilization while as I have shown (58) the rate of oxygen consumption decreases. No data are available on the effects of fertilization upon the permeability of the *Chaetopterus* egg. Decreased permeability to water is in some cases associated with anesthesia<sup>14</sup> and increased permeability with stimulation and increased activity.

This is probably interrelated with the more or less parallel changes in

<sup>12</sup> The unfertilized *Nereis* egg is comparatively highly gelated, as is the immature sea urchin egg, due perhaps to the large amount of water held at this time in the large immature nucleus.

<sup>13</sup> Hobson (17) also finds an increase following fertilization in *Psammechinus miliaris*.

<sup>14</sup> Depending however as Lucké (36) has shown in the case of the unfertilized *Arbacia* egg upon the ions in the medium, etc.

protoplasmic consistency.<sup>15</sup> As far as the data go with respect to oxygen-consumption rate, viscosity, and permeability, the unfertilized egg resembles in a general way a cell in a state of anesthesia, although the evidence from the rates of oxygen consumption is of the negative sort that inhibition does not appear to be caused by low oxygen-consumption rate as a limiting factor except perhaps in the case of the sea urchin egg.

There is possibly some danger of word play in distinguishing between release from inhibition on the one hand and stimulation on the other, but it seems to me profitable to make the distinction here, considering the former the result of the latter, and that it is necessary to regard the young resting egg cell as positively inhibited. Moderate anesthesia raises the threshold for stimulation but it does not abolish responsiveness. The immediate response of the unfertilized egg to the stimulation of the spermatozoon or a variety of agents is to abolish the condition of inhibition and transform to a normal (*i.e.* growing) cell. Specificity in fertilization, and related complications have to do with the nature of the stimulation itself, and the mechanism of activation; *i.e.*, the release from inhibition. I shall not attempt to bring this into the discussion.<sup>16</sup>

Wasteneys (56) used Loeb's methods for inducing what is regarded as a reversal<sup>17</sup> of activation of the *Arbacia* egg, and measured the effects on the rate of oxygen consumption. When eggs have been given preliminary parthenogenetic treatment, the development which ordinarily ensues when they are placed in sea water is prevented by NaCN or by chloral hydrate. Eggs so inhibited are now subject either to fertilization, or to artificial reactivation and will develop. Wasteneys shows that when eggs are "reversed" with NaCN, the oxygen-consumption rate reverts to the low prefertilization rate, and that when the eggs are

<sup>15</sup> Hobson (17) finds a changing permeability to water between fertilization and the first cleavage in the sea urchin egg which has interesting parallels in part to Heilbrunn's (15) corresponding consistency curve.

<sup>16</sup> Ralph Lillie (24) gives an excellent brief theoretical discussion of the nature of activation in the starfish egg.

<sup>17</sup> Frank Lillie (20, page 165) is of the opinion that this is an inhibitory effect rather than a true complete reversal of activation.

reactivated, it again rises to the rate of fertilized eggs. However, and this is important, the same developmental effects are caused by chloral hydrate, although this anesthetic does not lower the rate of oxygen consumption nearly to the same extent, nor nearly to the low level of the unfertilized eggs. Wasteneys concludes that the main factor concerned in bringing about the reversion is a suppression or inhibition of the developmental processes, and that lowering the rate of oxidation (NaCN) is merely one means of bringing this about. It is perhaps the means naturally operating in the unfertilized sea urchin egg. There is no reason to expect it to be the means in eggs generally.

The successful substitution of chloral hydrate and other anesthetics for cyanide or oxygen lack, to produce the same effect in a number of situations (*e.g.* see footnote 3), suggests that in them it is not the changed oxidation rate as such which is effective, but rather entrance into or liberation from a state of inhibition (whatever structure or condition of the protoplasm this state may imply) and that an induced "asphyxiation" anesthesia is merely one way of bringing about the anesthesia.

### *Energy Relations*

The rate of oxygen consumption is by no means a complete measure of the rate of oxidations in the egg. Anaerobic oxidations may in some cases play an appreciable part in the metabolic energy source. Loeb (28) found evidences of hydrolytic processes in the sea urchin egg, and Barron (4) has shown not only that the *Nereis* egg has a high tolerance for anaerobiosis but also (3) that its metabolic reaction to certain dyes supports the view that it carries out carbohydrate fermentations. The calorific quotients obtained with sea urchin eggs before and after fertilization by Meyerhof (41) and by Shearer (46) do not indicate any anaerobic source of heat nor any change at fertilization, and Runnström (44) has shown that the anaerobic reduction of methylene blue occurs with the same rapidity in fertilized and unfertilized eggs. But especially in an egg such as that of *Chaetopterus* which reduces its aerobic oxidation rate at fertilization, it is possible that anaerobic oxidations increase at fertilization so that the total oxidations may not decrease at this time when energy requirements would appear to be increased. Measurements of heat and metabolite

production may yet demonstrate this to be the case, and if so, using "oxidation" in the broad sense, generalization of Loeb's hypothesis may turn out to hold. But in view of the very high absolute rates of aerobic oxidation of the inhibited *Chaetopterus* and *Cumingia* eggs, together with the relations shown in Fig. 2, there is no basis for supposing so at the present time.

### *The Rate of Oxygen Consumption Per Unit Cell Surface*

Many eggs undergo a change in diameter, and therefore of cell surface, following fertilization. This has been best measured in the *Arbacia* egg, and the change in surface is very slight compared with the change in rate of oxygen consumption, which can in no wise be explained as due to or proportional to the change in surface area. In *Arbacia* the change in diameter is so slight that there has been dispute as to its direction. According to Glaser (10) the diameter decreases from 74 to 71.7 microns. Gray (12) has shown that cell division, with its sudden increase of surface, is not accompanied by any change in rate of oxygen consumption, and Warburg (52) has shown that the suppression of cell division by urethane does not appreciably effect the rate of respiration. Gray (13) has shown that in the growing trout embryo the rate of oxygen consumption is proportional to the wet weight of living embryo. The change in rate of oxygen consumption at fertilization, in those eggs in which the change is great, can be explained only by assuming a change in the arrangement or condition of the substances in the egg.

A comparison of the rates of oxygen consumption by the fertilized eggs of the several animal species shows that they are somewhat more nearly the same per unit volume of egg material than per unit cell surface. The unfertilized eggs differ widely on either basis. It is no doubt to be expected that, other things being equal, the rate of respiration per unit weight or volume will be greater when cells are small and have relatively more surface. The fertilized egg of *Arbacia punctulata* has a comparatively high rate per unit volume (Fig. 2) and is a comparatively small egg (74 microns diameter). But other factors enter, as the still smaller fertilized *Cumingia* egg (about 66 microns diameter) has a considerably lower rate (Fig. 2).



*The Effect of the Nuclear Resting Stage and the Cortical Change at Fertilization*

The eggs of *Chaetopterus* and of *Cumingia*, which I have found to decrease the rate of oxygen consumption following fertilization, both respire at a comparatively high rate in the resting stage before fertilization. They also both rest in the same nuclear stage of maturation, the metaphase of the first polar spindle. This stage (the metaphase) is an especially stable one in the mitotic cycle, and the possibility of a correlation between this stage and a high respiratory rate must be considered. The general evidence from most work which bears on the effect of the mitotic stage in the cleavage cycle is against any relationship (see Needham (40, vol. 2, page 641)), although it is possible that an effect might be of such brief duration during mitosis that it has not been detected, whereas the effect would cover an indefinite period of time in eggs which rest in this stage. I attempted to make measurements on another egg which rests in this same stage (*Cerebratulus*) but was unable to obtain satisfactory measurements because of the large amounts of jelly adhering to these eggs. However Runnström (44) reports that a few measurements on the eggs of *Ciona intestinalis* indicate a rise following fertilization. Morgan (39, page 62) states that this egg rests before fertilization in the metaphase of the first polar spindle. There is not therefore a consistent relation between this resting stage and a decrease in the rate of oxygen consumption following fertilization.

The relation between increase in rate of oxygen consumption and the cortical breakdown which attends fertilization and membrane formation in a number of eggs has entered into most attempts to explain the changes in the egg at fertilization, beginning with Loeb's early hypothesis. However, as Just points out, there is great variation among eggs in the extent to which this breakdown or disintegration takes place. Just (18, 19) relates this breakdown in *Arbacia* to the peak of heat production found immediately after fertilization by Rogers and Cole (43), and to the greater peak in oxygen-consumption rate reported in *Echinus* by Shearer (45). Just states (private communication) that the cortical changes at fertilization in *Cumingia*, *Chaetopterus*, and *Asterias* are rather slight compared with the changes

in *Arbacia* and *Nereis*. A certain amount of correlation therefore exists between the degree of cortical change and the increase in respiratory rate at fertilization, but it is not striking since the *Nereis* egg, which has the greatest and especially the longest duration of cortical change, increases its respiratory rate only very moderately, and then not to the high absolute rate (either per unit volume or per unit cell surface) of the resting unfertilized eggs of *Cumingia* and *Chaetopterus*. This correlation, like the comparison of surface and volume rates, and comparisons of the nuclear stages of resting eggs and the change in rate of respiration at fertilization, can be more profitably attempted when accurate absolute data are available from a greater variety of eggs.

*Discrepancies in Measurements on the Eggs of Arbacia punctulata, and the Relation of These to the Calorific Quotient*

The absolute rates of oxygen consumption per unit volume by eggs of the four sea urchins: *Arbacia pustulosa*, *Arbacia punctulata*, *Echinus miliaris*, and *Paracentrotus lividus*, as determined by Warburg, Shearer, Runnström, and myself agree quite closely (Fig. 2), although the large egg of *E. miliaris* (Shearer) with relatively less surface and probably more yolk in proportion respire at a slightly lower rate per unit volume than the small egg of *A. punctulata*. Tang's (47) values for *Arbacia punctulata* however are about three times as high as mine for the same species (Fig. 2). It is difficult to examine into the probable correctness of Tang's values because he gives very little data bearing on the question. His immediate interest is the rate of oxygen consumption by the unfertilized eggs as a function of the oxygen tension. Twenty-four measurements made on the unfertilized eggs in equilibrium with air show a range of threefold in absolute determination. He gives no data on the increase at fertilization, but states that, "the experiments showed fertilized eggs to have a respiration 5 times that of the unfertilized ones, confirming Warburg and Loeb and Wasteneys; . . . ." There is no indication whether the fivefold increase was observed from the lower, average, or higher level of the threefold span of rates of unfertilized eggs. Tang states that the rate of shaking was demonstrated adequate for maintaining gas equilibrium, but he does

not say what it was nor indicate that the other danger, namely that too rapid shaking abnormally increases the rate of respiration was realized or guarded against. In his work on the starfish egg (48) he used a rate of shaking so rapid (seventy complete oscillations per minute with 15 cm. amplitude) that in my experience it inevitably would have seriously damaged *Arbacia* eggs. It is not quite clear whether Tang used  $\frac{1}{2}$  cc. of egg suspension, or  $\frac{1}{2}$  cc. of eggs in 3 cc. suspension per manometer vessel. If the latter is the case the high concentration of eggs, requiring high shaking rate, would have tended to increase cortical damage.

Tang and Gerard (49) have more recently obtained absolute measurements of the rate of oxygen consumption by *Arbacia* eggs 95 per cent or more fertilized before being placed in the manometer vessels. The fertilized eggs are probably less subject to increased respiration rate by cortical damage than unfertilized eggs (see discussion earlier in this paper), and Tang and Gerard in this case find the average rate to be 3.5 times the average rate for unfertilized eggs found by Tang, instead of five times. In volume units this means a rate of 4.2 instead of 6.1 for the fertilized eggs (Fig. 2). They explain this discrepancy by stating that it is not entirely safe to compare values from one season to another. The methods were the same as in the preceding work. Eggs which exuded from a large number of gonads were filtered through cheese cloth. This is a dangerous procedure, because the respiratory rate of the *Arbacia* eggs may be altered when damage is not great enough to prevent fertilization and cleavage. It is by no means necessary, at least in the case of the unfertilized eggs, to cause complete cytolysis before greatly increasing the respiratory rate. Tang and Gerard used a rapid shaking rate, sixty-five complete oscillations per minute, with 10 cm. amplitude, which they state was just short of the rate which caused the fertilized eggs to be largely cytolized at the end of an experiment. They also found that oxygen-consumption rate varied with changed rate of shaking, although this could not be due to incomplete oxygen equilibrium because substituting oxygen for air did not increase the observed rate.

The precautions which I took in guarding against damage to the eggs on the one hand and inadequate gas exchange on the other, have been discussed in the fourth paper of this series (60) in conjunction

with my results on *Arbacia*. By way of summarizing, it can be said that the higher values of Tang, and of Tang and Gerard, especially the former, could be adequately accounted for in magnitude by the elevation of respiratory rate which a number of investigators have found to be caused in the sea urchin egg by even moderate cortical damage. If on the other hand their values, or one of them, is correct, my lower value would have to be explained by assuming inadequate gas exchange due to slower shaking. To the evidence earlier presented against this it should be added that in the same vessels, with the same depth of solution, the same rate of shaking, and even greater gas-exchange rate, gas equilibrium was adequately maintained when *Chaetopterus* eggs were used. The adequacy of gas exchange could be more certainly determined with the *Chaetopterus* egg due to its superior properties for the purpose.

So far as I have been able to determine, the numerous measurements of the rate of oxygen consumption which Loeb and Wasteney made on this egg were always in relative terms, so that no absolute measurements are afforded for comparison. The only other absolute measurements which directly correlate with the oxygen-consumption measurements are the heat-production measurements by Rogers and Cole (43). They give the rate of heat production per million eggs per hour, before fertilization as 0.08 calories, and after fertilization when the eggs have reached the two-cell stage as 0.52 calories. But since in their paper they do not state the temperature at which the eggs produced heat at this rate, the values are indeterminate. Dr. Cole informs me (private correspondence) that the measurements were made at the temperature of the running sea water in the laboratory, which he believes to have been in the neighborhood of 16–18°C.

If the gram calories of heat produced per unit amount of eggs per unit time is divided by the milligrams of oxygen consumed, the calorific quotient is obtained. Needham (40, page 651) gives the approximate theoretical calorific quotients for carbohydrate, fat, and protein metabolism respectively as 3.5, 3.3, and 3.2. Meyerhof's (41) values for the calorific quotient of *Paracentrotus lividus* eggs, unfertilized and at various stages of development, lie between 2.53 and 2.9, all lying under the theoretical. Shearer's (46) determinations on *Echinus miliaris* are somewhat higher: 3.07 before fertilization and 3.2 for the

first hour after fertilization. Relatively more heat was produced and the calorific quotients approximate more closely to the theoretical. If the calorific quotients are calculated, using Rogers and Cole's values for the heat production in *Arbacia punctulata*, and the oxygen-consumption measurements for the same species by Tang, Tang and Gerard, and myself, assuming Rogers and Cole's temperature to have been 17°C. and correcting all temperatures to 21° C. on the assumption that  $Q_{10} = 2$ , the results are rather anomalous. For the heat production I have taken the rate which holds constantly after the two-cell stage is reached. Before this stage the heat-production rate is considerably greater, and also therefore is the calorific quotient. Tang's values give a calorific quotient before fertilization of 2.9, after fertilization of 3.8. Tang and Gerard's later value for fertilized eggs gives a calorific quotient of 5.5. My values give a calorific quotient before fertilization of between 7 and 8.7 and after fertilization of 11.4. In other words, the rate of heat production is much too high to give a theoretical quotient with any of the oxygen measurements except Tang's high values for unfertilized eggs, which are almost certainly too high. My own values, which agree well with those obtained with a variety of sea urchin and other eggs, fit the least well. Anaerobic metabolism could account for a calorific quotient above the theoretical for the complete oxidation of carbohydrate, fat, or protein, but it is improbable that it could account for such great elevation of the quotient.<sup>18</sup> It therefore seems to me probable that Rogers and Cole's values for heat production are too high. Most of the factors which can so readily cause an increased rate of oxygen consumption, notably cortical damage, also cause increased heat production (Shearer (46)). If anaerobic metabolism is involved the effects might be greater in the case of heat. There is also the possibility, when measurements of heat production are made in a thermostat below room temperature, of slight influx of heat into the experimental vessel. Even with a differential method of calorimetry this is possible when the system is not entirely symmetric; *i.e.*, when the apparatus or treatment attached to the two Dewar flasks differs.

Needham (40, pages 652 and 658) has made an interesting calcula-

<sup>18</sup> Neither Meyerhof nor Shearer obtained enough heat production to account for any anaerobic oxidation.

tion of the calorific quotient using Shearer's data on *Echinus miliaris* for oxygen consumption and Rogers and Cole's values from *Arbacia punctulata* for heat production. Needham did this in the absence at the time of oxygen-consumption data on *Arbacia punctulata*, and regards the calculation, involving as it does different species, as merely an interesting "feeler." The interesting thing about this calculation is that the values for the calorific quotient turn out to be 3.51 for the unfertilized eggs, and 3.7 for the fertilized eggs in the two-cell stage. The first of these values is almost exactly theoretical for carbohydrate metabolism (which is expected here from the respiratory quotient values (46)) and the second is not much above. In other words, it is the most perfect fit, compared with Meyerhof's and Shearer's determinations. This appears in a sense to afford a rather striking confirmation of Rogers and Cole's values. However, since Shearer's measurements were made at 14.5°C. and Rogers and Cole's at about 17°, when correction is made for temperature the quotients are reduced. Further, the comparison was on the basis of oxygen and heat per million eggs. One *Echinus miliaris* egg has four times the volume of one *Arbacia punctulata* egg (see appendix). There is therefore no reasonable basis for comparison on the basis of equal number of eggs. If the calorific quotients are calculated with temperature corrections and on the basis of heat and oxygen per unit volume of cells, they become: before fertilization, 12.6, after fertilization in the two-cell stage, 14.4. These are the highest values of all, and in so far as comparison between species is tenable, add to the probability that Rogers and Cole's heat determinations are too high. Per million eggs, Rogers and Cole's values for heat production are only somewhat higher than those of Meyerhof and Shearer. But per unit volume of egg material they are more than five times as high as Shearer's, for eggs in the two-cell stage.

Needham (40, page 658) points out that both Meyerhof and Shearer found an increasing rate of heat production, closely following the increasing rate of oxygen consumption, after fertilization, giving thus a constant calorific quotient, while Rogers and Cole find constant heat production from the two- to the eight-cell stage. An inspection of Table V in the fourth paper of this series (60) shows that the rate of oxygen consumption is already rising in *Arbacia punctulata* within the

2nd hour after fertilization,<sup>19</sup> during which time and after which Rogers and Cole find constant heat production. This implies a constantly decreasing calorific quotient between the two- and eight-cell stages which would be very interesting if the absolute value of the quotient were more reasonable.

It is difficult to obtain accurate absolute metabolic rate measurements with the delicate eggs of *Arbacia punctulata*. Measurements should be repeated with great attention to the condition and the treatment of the eggs, as well as to the method of measurement.

#### SUMMARY

1. The rate of oxygen consumption by eggs may not merely undergo no change at fertilization, as in the case of the starfish, but it decreases to about half in *Chaetopterus* and in *Cumingia*.

2. The absolute rate of oxygen consumption in mm.<sup>3</sup> O<sub>2</sub> per hour per 10 mm.<sup>3</sup> eggs differs widely in several species of unfertilized eggs. It is very low in the sea urchin, intermediary in *Nereis*, and high in *Chaetopterus* and *Cumingia*. The range for these eggs is approximately 0.4 to 3.1 mm.<sup>3</sup> O<sub>2</sub> per hour per 10 mm.<sup>3</sup> eggs at 21°C., in the ratio of about 1:8.

3. The absolute rates of oxygen consumption by the same fertilized eggs are much more nearly the same. They lie within the range 1.3 to 2.0 mm.<sup>3</sup> O<sub>2</sub> per hour per 10 mm.<sup>3</sup> eggs at 21°C., in the ratio of approximately 1:1.5. Within this same range lie the values obtained by a number of investigators using a variety of eggs of invertebrates from several phyla. *Amoeba proteus* and frog skin also are within this range (see Fig. 2).

4. The changes in rate of oxygen consumption at fertilization by the different species of eggs, differing both in direction and magnitude, appear to be such as to bring the rate, when development is initiated, to about the same rate, which is also the rate of other comparable normally growing cells.

5. The direction and magnitude of the change in rate at fertilization therefore appears in the cases cited to be primarily a function of the absolute rate of oxygen consumption by the unfertilized eggs,

<sup>19</sup> This agrees with the measurements of Loeb and Wasteneys (28, page 29).

which are characterized in their peculiar inhibited condition, among other things, by a wide range of respiratory rates.

6. It is not to be supposed that this range of rates will apply at all universally to eggs, especially to eggs of extremes in proportional content of inert materials, such as large yolky eggs. Fish and amphibian eggs for example respire at a much lower rate per unit volume. The effect on surface: volume ratios attending extremes of cell size might also be expected to shift the absolute rate.

7. The absolute rate of oxygen consumption by the eggs of the alga *Fucus vesiculosus* is considerably higher than the rates of the animal eggs measured. It is of the same order of magnitude as the rates of several other small-celled algae, which respire at a greater rate per unit volume than most non-motile animal cells.

8. The comparatively high rates of oxygen consumption by the inhibited (unfertilized) eggs of *Chaetopterus* and *Cumingia* are not directly associated with nuclear or morphological activity of the cell since they continue at the high rate for hours after cessation of the brief initial nuclear activity, which takes place when the eggs are placed in sea water.

9. It is concluded that the rate of oxygen consumption is not necessarily and probably not generally the limiting factor which causes inhibition of the unfertilized egg. Increase in rate of oxygen consumption is not directly related to the initiation of development, in general, nor even necessarily concomitant. It is not improbable that the low rate of oxygen consumption is an immediate part of the cause of inhibition of the unfertilized sea urchin egg, but this is a special case.

10. This thesis, that the rate of oxygen consumption is not necessarily nor ordinarily the limiting factor in the inhibition of the unfertilized egg, and conversely that increase in the rate of oxygen consumption is not usually the essential feature of fertilization, is quite in agreement with the general relations between the rate of oxygen consumption on the one hand and anesthesia, growth, and development on the other in fertilized eggs and other organisms.

11. This conclusion is opposed to Loeb's explanation of the essential feature of fertilization, as an increase in oxidation<sup>2</sup> rate or more strictly to generalization of his hypothesis to include eggs other than those of the sea urchins (or of other similar special cases which may be dis-



covered). It extends to fertilization (the initiation of development) his and Wasteneys well established conclusion that "oxidation<sup>2</sup> is not the independent variable in development."

12. It is suggested that the crux of the problem of fertilization lies in the nature of the inhibition of the unfertilized egg. Certain similarities between this condition, arrived at spontaneously in the case of the egg cell, and the condition of cells in narcosis or anesthesia are pointed out.

13. Although the rate of oxygen consumption by the unfertilized eggs of *Chaetopterus* and *Cumingia* cannot be regarded as the limiting factor which causes the inhibition of the eggs, in these and other cases with different absolute rates, it appears highly probable that the rate of oxygen consumption is in some way, at present obscure, tied up with or related to the condition of inhibition. This seems probable especially in view of the sharp change in rate which in most cases immediately attends cessation of the inhibition, but the relationship may be a non-causal one, as in narcosis.

14. It must be borne in mind that oxygen consumption is not necessarily a complete measure of oxidation, and that other measures such as of heat and metabolite production are necessary before the complete amount of oxidation is known. When these are completely worked out, if free energy relations are known, it is probable that more direct and inclusive relations may be found between oxidation, growth, development, and anesthesia. Generalization of Loeb's hypothesis, using "oxidation" in the broad sense might then turn out to hold, with fertilization fitting into the general scheme, but there is no basis for it at the present time.

I am much indebted to Professor W. J. Crozier for advice and criticism, especially in the earlier stages of this investigation, and for reviewing the manuscript.

## APPENDIX

### *Derivation and Conversion of Data Plotted in Fig 2.*

The data given in graphical form in Fig. 2 have been taken from a number of sources which often have involved conversion of units and in some cases involve assumptions which are given here. Runnström

(44) gives data for a number of controls in his experiments on *Paracentrotus lividus* which involve the volume of egg suspension used, the per cent by volume of eggs in the suspension as determined by centrifuging in calibrated blood tubes, the temperature, the duration of measurement, and the total cubic millimeters of oxygen consumed. I have taken only data on normal control runs on fertilized eggs from his paper. The measurements which are accompanied with sufficient data for the purpose, when converted to the absolute rate  $\text{mm.}^3 \text{ O}_2$  per hour per 10  $\text{mm.}^3$  eggs, are as follows:

	°C.				
Table 2 a,	23	105 minutes measured	52 $\text{mm.}^3$ eggs in 2 cc. suspension		2.91
" 3 c,	23	105 " "	32 " " " 1 " "		2.71
" 4 c,	23	75 " "	84 " " " 2 " "		3.92
" 11 c,	20.5	120 " "	76 " " " 2 " "		1.5
" 13 c,	21	120 " "	58 " " " 2 " "		2.1
" 22 a,	20.5	90 " "	84 " " " 2 " "		1.3
" 25 a,	25	140 " "	62 " " " 2 " "		2.76

Before averaging the results I have multiplied the values obtained at 25°C. by 0.75 and those at 23° by 0.85 as approximate temperature corrections to 21°C. on the assumption that  $Q_{10} = 2$ . Leaving unchanged the values obtained at 20.5°C., the average value then becomes, as of 21°C., 2.16, which is plotted in Fig. 2. It should be noted that these measurements run for from 75 to 140 minutes, and since the respiratory rate is increasing with time after fertilization, the value taken is undoubtedly somewhat higher than it would be more immediately after fertilization. Considering this, it agrees very well with Warburg's value for the same species.

Fauré-Fremiet's (9) determinations of the rate of oxygen consumption by the fertilized and unfertilized eggs of *Sabellaria alveolata*, at 20°C., are expressed in terms of weight of eggs and weight of oxygen. These have been converted to volume units by assuming a density of the eggs of 1.04. The rates then become for fertilized eggs 2.05  $\text{mm.}^3 \text{ O}_2$  per hour per 10  $\text{mm.}^3$  eggs, and 1.83 for unfertilized eggs at 20°C.

Shearer (46) gives the rate of oxygen consumption per hour per million eggs, fertilized and unfertilized, of *Echinus miliaris*. Professor J. Gray has kindly referred me to Hobson's (17) measurements<sup>20</sup>

<sup>20</sup> An earlier paper (16) indicates a smaller size.

of the volume of this egg, and has placed me in communication with Professor Hobson who has been good enough to inform me that there is appreciable variation in the size of the egg, but that on the average the volume of an unfertilized egg is about  $850,000\mu^3$ . This permits conversion of Shearer's data to volume units. Shearer's measurements were made at  $14.5^\circ\text{C}$ . and a comparatively large correction is therefore necessary to convert to values corresponding to  $21^\circ\text{C}$ . This was done, assuming  $Q_{10}$  to equal 2, and the values become for the unfertilized eggs  $0.28\text{ mm}^3\text{ O}_2$  per hour per  $10\text{ mm}^3$  eggs, and for fertilized eggs 1.59, at  $21^\circ\text{C}$ .

The values for *Amoeba proteus* are taken directly from Emerson's paper (8), in which the same units were employed which I have used. I have also taken the value for Adolph's (1) measurements on fresh frog skin from Emerson's paper since here Adolph's values have been converted to volume units from weight units by assuming a density of 1. Warburg (55) states that at  $23^\circ\text{C}$ ., an amount of unfertilized eggs of *Paracentrotus lividus* which contain 20 mg. egg nitrogen consume  $10\text{--}14\text{ mm}^3\text{ O}_2$  in 20 minutes. In an earlier paper (53) he also states that 1 cc. of centrifuged *Paracentrotus* eggs contain 20 mg. egg nitrogen. Therefore  $10\text{ mm}^3$  unfertilized eggs consume oxygen at the average rate of  $0.36\text{ mm}^3$  per hour at  $23^\circ\text{C}$ . Multiplying this by 0.85 the appropriate value for  $21^\circ\text{C}$ . becomes 0.31. He also states that the fertilized eggs, beginning 10 minutes after fertilization, consume oxygen at six times this rate, or at about  $1.86\text{ mm}^3\text{ O}_2$  per hour per  $10\text{ mm}^3$  eggs at  $21^\circ\text{C}$ .

In 1908 Warburg (50) found that the unfertilized eggs of the European *Arabacia pustulosa* consume  $0.05\text{--}0.06\text{ mg. O}_2$  per hour per 28 mg. egg nitrogen at  $20.5^\circ\text{C}$ ., and that the fertilized eggs consume oxygen at six to seven times this rate. I have converted mg.  $\text{O}_2$  to  $\text{mm}^3\text{ O}_2$ , and then in order to get an approximate value in terms of volume units I have taken some license and assumed that the same ratio exists between egg volume and egg-nitrogen content as in *Paracentrotus*; i.e., that 20 mg. nitrogen represents 1 cc. eggs. If this is true the middle value at  $20.5^\circ\text{C}$ . becomes  $1.68\text{ mm}^3\text{ O}_2$  per hour per  $10\text{ mm}^3$  eggs.

Tang's (47) average value for the rate of oxygen consumption by unfertilized eggs of *Arbacia punctulata*, at  $24.7^\circ\text{C}$ . is  $33.6\text{ mm}^3\text{ O}_2$

per hour per million eggs. The fertilized eggs were found to consume oxygen at five times this rate. Assuming a diameter of 74 microns for the egg, and multiplying by 0.77 as an approximate temperature correction to 21°C., the converted values become 1.22 and 6.1 mm.<sup>3</sup> O<sub>2</sub> per hour per 10 mm.<sup>3</sup> eggs at 21°C.

Tang and Gerard (49) find an average rate, at 25°C., of 118 mm.<sup>3</sup> O<sub>2</sub> per hour per million fertilized eggs of *Arbacia punctulata*. Converting to volume units and to 21°C., this becomes 4.2 mm.<sup>3</sup> O<sub>2</sub> per hour per 10 mm.<sup>3</sup> eggs.

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## THE KINETICS OF PENETRATION

### V. THE KINETICS OF A MODEL AS RELATED TO THE STEADY STATE

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A previous article<sup>1</sup> describes accumulation in models, where potassium has the appearance of diffusing in against a gradient. This raises an interesting question in kinetics.

The situation is shown in an experiment with 0.05 M KOH shaken up with a small quantity of a non-aqueous mixture of 70 per cent guaiacol + 30 per cent *p*-cresol (this will be called G. C. mixture). Practically all the KOH was thereby converted to organic salts. After separation from the non-aqueous phase the aqueous solution was allowed to flow in a steady stream through the model (*A*, Fig. 1) where it came in contact with a non-aqueous layer (*B*) of G. C. mixture. Potassium penetrated through *B* into *C* (which consisted at the start of distilled water through which bubbled a steady stream of CO<sub>2</sub>) and accumulated there in the form of KHCO<sub>3</sub>.<sup>2</sup> All three layers were stirred mechanically.

The formation of KHCO<sub>3</sub> in *C* raised the osmotic pressure so that water entered and the volume of *C* increased. A state was eventually reached where water and potassium entered in a constant ratio and in consequence the concentration of potassium in *C* remained approximately constant while *C* continued to increase in volume. This will be called the steady state.

The only cases of this sort suitable for kinetic studies are those where the volume of *C* is so large and the increase so gradual that reasonably accurate measurements of volume are possible. It hap-

<sup>1</sup> Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1931-32, **15**, 667.

<sup>2</sup> We may compare *C* to the sap of a living cell and call it "artificial sap:" *A* then represents the external solution and *B* the non-aqueous protoplasmic surface.



pens that only one of these<sup>3</sup> (Exp. 64) was carried out in a manner suited to our purpose (since in the others the  $\text{CO}_2$  was not bubbled from the very start).

In this case the external solution in the steady state contained 0.041 M potassium combined with guaiacol and *p*-cresol plus 0.009 M  $\text{KHCO}_3$  (due to the diffusion<sup>1</sup> of  $\text{CO}_2$  or  $\text{HCO}_3$  from *C* into *A*). The solution in *C* contained 0.63 M potassium, practically all as  $\text{KHCO}_3$  (with a very little potassium combined<sup>4</sup> with guaiacol, *p*-cresol, and hydroxyl). All of this potassium (except a small amount of KOH) passed from *A* into *B* as potassium guaiacolate (which we may call KG) and as potassium *p*-cresolate: since the latter closely resembles

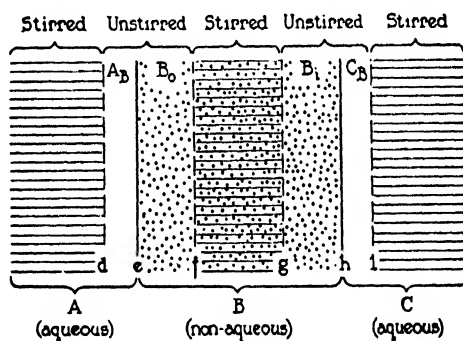


FIG. 1. Diagram of layers in the model. The aqueous phase *A* has an unstirred layer which is represented between *d* and *e*: from *e* to *f* is the corresponding unstirred layer in the non-aqueous phase *B*. Similar layers are present at the boundary between the non-aqueous phase *B* and the aqueous phase *C*.

the former we may for convenience regard them as equivalent and say that all the potassium enters *B* as KG.

To understand the behavior of potassium under these conditions let us consider the factors that influence rates of penetration. These factors have been discussed by Irwin<sup>5</sup> in connection with the plasma

<sup>3</sup> This is Exp. No. 64 of a preceding paper (1). The external pH value was about 9.1 and the internal about 7.6 in the steady state.

<sup>4</sup> A saturated solution of guaiacol in water at 25°C. is stated to be 0.15 M and that of *p*-cresol at 20°C. to be 0.18 M. Cf. Seidell, A., Solubility of inorganic and organic compounds, New York, D. Van Nostrand Co., Inc., 2nd edition, 1919, 278 and 309.

<sup>5</sup> Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1927-28, **25**, 127; 1931-32, **29**, 993.

membrane. Overton had said that the rate of penetration depends on the partition coefficient at the outer surface of the plasma membrane, but Irwin stated that other coefficients, especially at the inner surface, may be of equal importance. This conception was embodied in the "multiple partition coefficient theory."<sup>6</sup>

In dealing with models Irwin stated that the rate of penetration of dye depends on its rate of diffusion across the non-aqueous layer<sup>7</sup> which in turn depends in part on the partition coefficients at the two phase boundaries. Hence, other things being equal, penetration is rapid<sup>8</sup> when the external partition coefficient is high and the internal low or when the dye on reaching the sap is converted to a form with a low partition coefficient.<sup>9</sup> The rate is regarded as a function of such factors as (1) concentrations in the aqueous solutions, (2) the partition coefficients, (3) the concentration gradient<sup>9</sup> in the non-aqueous layer, and (4) the diffusion coefficients, the nature of this function being left to future investigation to determine.<sup>10</sup>

Northrop,<sup>11</sup> in a paper on the permeability of dried collodion membranes, has given the following formula:  $Q = \frac{tDA(S_1C_1 - S_2C_2)}{h}$ ,

where  $Q$  is the amount passing through,  $A$  is the area,  $h$  the thickness of the collodion membrane,  $S_1$  and  $S_2$  are the partition coefficients at the outer and inner surfaces,  $C_1$  and  $C_2$  are the concentrations in the external and internal solutions,  $D$  is the diffusion coefficient in the collodion, and  $t$  is time.

The relation of this formula to activities becomes evident from the following treatment, kindly suggested by D. A. MacInnes. If we consider a non-electrolyte diffusing through a membrane of unit area and thickness we may write

$$v = u \frac{\partial \mu}{\partial x}$$

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<sup>6</sup> The term partition coefficient was later replaced by absorption coefficient as in addition to solubility chemical action and other processes may be involved. Cf. Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1931-32, **29**, 993.

<sup>7</sup> Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1928-29, **26**, 125; *J. Gen. Physiol.*, 1928-29, **12**, 407.

<sup>8</sup> Irwin, M., *J. Gen. Physiol.*, 1928-29, **12**, 407.

<sup>9</sup> Cf. Irwin, M., *J. Gen. Physiol.*, 1928-29, **12**, 163, 164, 408.

<sup>10</sup> Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1931-32, **29**, 1234.

<sup>11</sup> Northrop, J. H., *J. Gen. Physiol.*, 1928-29, **12**, 435.

where  $v$  is the velocity and  $u$  the mobility of the solute molecule,  $\mu$  is the thermodynamic potential and  $x$  is distance in the membrane. This may be rewritten as

$$v = u RT \frac{\partial \ln a}{\partial x}$$

where  $a$  is the activity. Since  $\frac{\partial \ln a}{\partial x} = \frac{1}{a} \frac{\partial a}{\partial x}$  we have

$$v a = u RT \frac{\partial a}{\partial x}$$

In the steady state the activity gradient in the membrane is linear, as shown by the straight line in Fig. 2, and we may write<sup>12</sup>

$$v a = u RT (a_1 - a_2)$$

Putting  $u RT = D$  (the diffusion constant) and writing  $c\gamma$  in place of  $a$  we have

$$vc\gamma = D(c_1\gamma - c_2\gamma)$$

If  $\gamma$  (the activity coefficient) be the same throughout the membrane it cancels out<sup>13</sup> so that we have

$$vc = D(c_1 - c_2)$$

where  $c_1$  and  $c_2$  are the concentrations corresponding to  $a_1$  and  $a_2$ . Since  $vc$  is the concentration of molecules multiplied by the velocity of the molecule it is equivalent to the quantity  $Q$  passing through any plane such as  $F$  (Fig. 2) in unit time, thus giving

$$\frac{\partial Q}{\partial t} = D(c_1 - c_2) = D \frac{\partial c}{\partial x}$$

Since  $\frac{\partial c}{\partial x}$  has the same value at  $F$  and at  $G$  the value of  $\partial Q$  is the same at both

<sup>12</sup> According to Guggenheim (Guggenheim, E. A., *J. Am. Chem. Soc.*, 1930, **52**, 1315) a similar equation applies to a single uni-univalent salt, but  $\frac{1}{D} = \frac{1}{2} \left( \frac{1}{D^+} + \frac{1}{D^-} \right)$  where  $D^+$  and  $D^-$  are the diffusion constants of the ions.

<sup>13</sup> If  $\gamma$  should not cancel out a correction must be applied but this will be small when the difference in  $\gamma$  is small, as would ordinarily be the case with non-aqueous media.

places and as the quantity of diffusate entering the region between  $F$  and  $G$  is the same as the quantity leaving it the concentration in this region remains constant. Hence the gradient in  $B$  is linear only when the concentration at the surface between  $B$  and  $C$  is constant which can only be the case when it is constant in the layer of  $C$  lying next to the surface, and this is evidently a necessary condition for the steady state (this condition is found in the model<sup>1</sup> in the steady state).

This is not true when the concentration gradient can be represented by the curved line in Fig. 2 for then  $\frac{\partial c}{\partial x}$  and  $\partial Q$  are greater at  $F$  than at  $G$ . But if the curvature be not too great and the thickness of the membrane constant, we may use the formula  $\frac{dQ}{dt} = D(c_1 - c_2)$  as an approximation.<sup>14</sup>

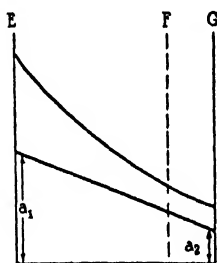


FIG. 2. Diagram to illustrate conditions in a membrane in whose surface the activities are  $a_1$  and  $a_2$  (the corresponding concentrations being  $c_1$  and  $c_2$ ). As shown by the ordinates  $a_1$  is greater than  $a_2$ . The ordinates for the curved line are made greater merely for convenience.

If the partition coefficients at  $E$  and  $G$  be  $S_1$  and  $S_2$  so that  $S_1 = c_1 \div c_A$  and  $S_2 = c_2 \div c_C$  ( $c_A$  and  $c_C$  being the concentrations in  $A$  and  $C$ ) we have

$$\frac{dQ}{dt} = \frac{D}{h} (S_1 c_A - S_2 c_C)$$

and

$$Q = \frac{D A t (S_1 c_A - S_2 c_C)}{h}$$

In many cases, as Irwin<sup>6</sup> has pointed out, it is desirable to replace the partition coefficient by an absorption coefficient on account of chemical action or other complications.

<sup>14</sup> The thinner the membrane the better the approximation.

These conceptions<sup>15</sup> may be applied to the present case since, despite the mechanical agitation, there are unstirred<sup>16</sup> layers at each interface. Movement through these layers is slow since it depends on diffusion. Such layers are shown in the diagram (Fig. 1).

As the viscosity of the G.C. mixture is greater than that of water the layers  $B_s$  and  $B_i$  are thicker than the corresponding aqueous layers  $A_B$  and  $C_B$ .

There are concentration gradients in all the layers but those in the stirred layers are small.

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<sup>15</sup> These conceptions have been used in setting up equations for penetration (Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1928-29, **26**, 192; *J. Gen. Physiol.*, 1929-30, **13**, 261).

<sup>16</sup> If we have to do with immiscible phases it is evident that no amount of stirring will produce in the interface itself any motion perpendicular to the interface. However, as we go away from the interface such motion will increase and will reach its maximum in the main body of the liquid. The entire layer in which such motion is submaximal may be regarded as practically equivalent to a thinner layer with no component of motion perpendicular to the interface which may be called the unstirred layer (in this layer there will be motion parallel to the interface but this will not directly aid the movement of substances from one phase to the other). The thickness of the unstirred layer varies with the rate of stirring but it has been found experimentally by various observers that with the most vigorous stirring (short of breaking the interface) such layers are thick enough to be the controlling factors in the passage of substances and the flow of heat from one phase to another.

Lewis and Whitman (Lewis, W. K., and Whitman, W. G., *Ind. and Eng. Chem.*, 1924, **16**, 1215) state that "in the main body of either liquid or gas, except under special conditions which will not be considered here, mixing by convection is so rapid that the concentration of the solute is essentially uniform at all points. On the other hand, the surface films are practically free from convection currents and consequently any transfer of solute through these films must be effected by the relatively slow process of diffusion. These films therefore offer the controlling resistances to transfer of material from one phase to another."

For a recent review of the literature see Davis, H. S., and Crandall, G. S., *J. Am. Chem. Soc.*, 1930, **52**, 3757. These authors state that in well stirred water microscopic observation shows particles on the surface and for about 0.04 cm. beneath it to have little or no motion perpendicular to the air-water surface. Such layers may be thicker in guaiacol, owing to its higher viscosity (22).

See also Whitman, W. G., and others in the Absorption symposium, *Ind. and Eng. Chem.*, 1924, **16**, 1215; also Whitman, W. G., Long, L., and Wang, H. Y., *Ind. and Eng. Chem.*, 1926, **18**, 363.

Potassium diffuses chiefly as KG (with some KOH) until it reaches *C* where it is mostly changed to  $\text{KHCO}_3$  in which form it diffuses in the layer  $C_B$ . There is also some KG and KOH in this layer; the situation may be explained as follows.

Assuming (as is customary) that the very thin films adjoining the interface between *B* and *C* are in approximate equilibrium<sup>17</sup> we may write<sup>18</sup>  $(KG)' = (C_p)(KG)$ , where  $C_p$  is the partition coefficient for activities,  $(KG)'$  is the activity of undissociated KG in the inner surface of *B*, and  $(KG)$  its activity in the outer

surface of  $C_B$ . We then have  $(KG)' = \frac{(K)(G)}{k}$ , where  $k$  is the dissociation constant of KG in *C* and  $(K)$  and  $(G)$  are activities in the outer surface of  $C_B$ . Hence we may write

$$(KG)' = C_p(KG) = \frac{C_p(K)(G)}{k}$$

Putting  $\frac{C_p}{k} = C_0$  we have

$$(KG)' = C_0(K)(G)$$

Now in *B*, KG is mostly undissociated since the dielectric constant of the solvent is low<sup>1</sup> but in *C* it is practically all dissociated and the value of  $K^+$  in *C* may be much greater than that of  $G^-$ .<sup>19</sup> Hence the concentration of potassium in *B* may be exceedingly small and that in *C* may be high but in spite of this potassium will continue to move from *B* into *C* because the undissociated KG in *B* is not in equilibrium with that in *C*. We shall see later on (p. 548) that on shaking up 0.63 M  $\text{KHCO}_3$  with G.C. mixture there is only 0.0062 M potassium in the latter: hence when *B* contains more than this potassium will move into *C* under such circumstances.

Let us now consider the situation with reference to  $\text{OH}^-$  and to  $\text{HCO}_3^-$ . This is probably not very different from that where guaiacol is used in place of the G.C. mixture. If this were the case we could proceed as follows. Under the conditions of the experiment the concentrations of  $\text{H}_2\text{O}$ ,  $\text{CO}_2$ , and guaiacol

<sup>17</sup> By this is meant that the concentration in the film in the outer surface of *B*, is approximately what would be found in *B* if *B* were shaken up with a relatively large volume of *A* and allowed to come to equilibrium.

<sup>18</sup> According to Lewis and Randall, when the activities in both phases are referred to the same standard state we may write  $(KG)' = (KG)$ . Cf. Lewis, G. N., and Randall, M., *Thermodynamics*, New York, McGraw-Hill Book Co., Inc., 1923, 257. See also Harned, H. S., in Taylor, H. S., *A treatise on physical chemistry*, New York, D. Van Nostrand Co., Inc., 2nd edition, 1931, 1, 762.

<sup>19</sup> See Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1931-32, 15, 682 (Table II).

(HG) remain approximately constant. If this be true of their activities we may put (following the procedure of L. G. Longworth)

$$\frac{(G)(H)}{(HCO_3)(H)} = \frac{k_{HG}(HG)}{k_{H_2CO_3}(H_2CO_3)} \quad \text{and} \quad \frac{(G)(H)}{(OH)(H)} = \frac{k_{HG}(HG)}{k_w}$$

where  $k_{HG}$  and  $k_{H_2CO_3}$  designate dissociation constants,  $k_w$  the water constant, and the brackets denote activities. Hence we may put  $G = C_8(OH) = C_9(HCO_3)$ , where

$$C_8 = \frac{k_{HG}(HG)}{k_w} \quad \text{and} \quad C_9 = \frac{k_{HG}(HG)}{k_{H_2CO_3}(H_2CO_3)}$$

Substituting these values in the equation

$$(KG)' = C_0(K)(G)$$

we have

$$(KG)' = C_0 C_8 (K)(OH) = C_0 C_9 (K)(HCO_3)$$

It follows that if we increase (K) (e.g. by adding KCl) or (G) there will be an increase in (KG)' and any increase in (HCO<sub>3</sub>) or (OH) will be accompanied by an increase in (G): likewise doubling (K) and (G) simultaneously will multiply (KG)' by 4 unless other factors intervene.<sup>20</sup>

With vigorous stirring such as occurred in this experiment the rate of penetration depends primarily on the rate of diffusion in the unstirred layers. Experiments with the diffusion apparatus of Northrop and Anson<sup>21</sup> show that KG passes so much more quickly through the aqueous than through the non-aqueous unstirred layers that the effect of the former may be neglected.<sup>22</sup> We need therefore consider

<sup>20</sup> Osterhout, W. J. V., *Biol. Rev.*, 1931, **6**, 369. Unpublished experiments indicate that other factors do intervene.

<sup>21</sup> Cf. Northrop, J. H., and Anson, M. L., *J. Gen. Physiol.*, 1928-29, **12**, 543. Experiments carried out by W. M. Stanley show that the diffusion constants of KG and NaG are about twelve times as great in water as in G.C. mixture (this will be discussed in a later paper).

<sup>22</sup> The aqueous layers are thinner due to the smaller viscosity. The viscosity of the G.C. mixture is higher and the layers consequently thicker than in the aqueous solutions. At 30°C. the viscosity of guaiacol in G.C.S. units is 0.0445 (Landolt, H., and Börnstein, R., *Physikalisch-chemische Tabellen*, Berlin, Julius Springer, 5th edition, (Roth, W. A., and Scheel, K.), 1931, suppl. vol. **2**, 102) while

only the diffusion in the non-aqueous layers,  $B_o$  and  $B_i$ . Since these layers are very thin<sup>14</sup> we may regard the concentration gradient as approximately linear. There is presumably a very thin film constituting the outer surface of  $B_o$  (not more than a few molecules in thickness), which is approximately in equilibrium with the corresponding film of the external solution;<sup>17</sup> the concentration of undissociated<sup>23</sup> KG in the film at the outer surface of  $B_o$  may be called  $K_o$ . The corresponding concentration at the inner surface of  $B$  we may call  $K_i$ . Hence we may designate the gradient<sup>24</sup> as  $K'_o - K'_i$  and (in accordance with the conceptions already discussed) regard  $\frac{dQ}{dt}$  as proportional to  $K'_o - K'_i$ .

The activity coefficient of KG in  $B$  cannot be far from unity and when we have pure guaiacol in  $B$ ,  $K'_o$  is approximately proportional (p. 535) to  $(K_o)(G_o)$  and  $K'_i$  to  $(K_i)(G_i)$ , where the subscripts  $o$  and  $i$  refer to activities of the potassium and guaiacol ions in the outside solution ( $A$ ) and the inside solution ( $C$ ) respectively. This will be approximately true in the G.C. mixture so that  $\frac{dQ}{dt}$  is approximately proportional to  $(K_o)(G_o) - (K_i)(G_i)$  and likewise<sup>25</sup> to  $(K_o)(OH_o) - (K_i)(OH_i)$  and to  $(K_o)(HCO_{3o}) - (K_i)(HCO_{3i})$ .

It follows that if  $K_i$  be increased by adding  $KHCO_3$  to  $C$  the value of  $\frac{dQ}{dt}$  would be decreased. This was done in the present experiment, as described in a former

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that of water is 0.0079 (Landolt, H., and Börnstein, R., *Physikalisch-chemische Tabellen*, Berlin, Julius Springer, 5th edition, (Roth, W. A., and Scheel, K.), 1923, 1, 135). According to measurements made in this laboratory by W. M. Stanley, guaiacol has a lower viscosity than guaiacol saturated with water, but the latter value is only slightly lower than that of G.C. mixture saturated with water (the last increases with increasing concentration of KG).

<sup>23</sup> Since we suppose that there is very little dissociation in the G.C. mixture, owing to its low dielectric constant (1), the value of  $K'_o - K'_i$  will not be much affected by neglecting the ionized portion.

<sup>24</sup> This gradient is the sum of the gradients in  $B_o$  and  $B_i$ . To this must be added any gradient in the stirred layer in  $B$ , but this is probably negligible.

<sup>25</sup> This has been employed in a previous paper (20) in using the equations for penetration elsewhere developed (Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 261).



paper.<sup>26</sup> In the steady state *C* contained 0.63 M  $\text{KHCO}_3$  (plus a small amount of guaiacol, KOH, and KG). Enough  $\text{KHCO}_3$  was then added to *C* to raise the concentration of  $\text{KHCO}_3$  to 1.1 M. The value of  $\frac{dQ}{dt}$  then fell off markedly.

This is borne out by shaking up G.C. mixture with aqueous solutions of KG and determining the value of KG in *B*: we find that this is increased by adding  $\text{KHCO}_3$  or KCl to the aqueous solution. This is not a simple salting out effect, for the addition of  $\text{NaHCO}_3$  or NaCl does not produce the same result (this will be discussed in a later paper).

As we are primarily interested in the rate of penetration of potassium regardless of the form it assumes in *C* we shall for convenience designate the total potassium in *C* (*i.e.*  $\text{KG} + \text{KHCO}_3 + \text{KOH}$ ) as  $\text{K}_i$  (this is practically equivalent to the concentration of  $\text{K}^+$ ). The corresponding concentration in *A* will be called  $\text{K}_o$ .

It may be added that there is an outward movement of potassium (chiefly as  $\text{KHCO}_3$ ), but as this appears to be relatively small (except in the later stages) it will be neglected.<sup>27</sup>

### *The Volume Curve*<sup>28</sup>

As more and more potassium enters *C* the osmotic pressure will more and more exceed that of *A*: hence water will enter more and more rapidly so that the greater the volume the greater its increase in unit time. This recalls the growth of a sum at compound interest which is expressed by putting  $M_t = M_o e^{k_o t}$ , where  $M_o$  is the sum at the start,  $k_o$  is a constant, and  $t$  is time. We find that the time

<sup>26</sup> Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1931-32, **15**, 677 (Exp. 64, Table I and Fig. 5).

<sup>27</sup> The ratio (conc. of potassium in G.C. mixture)  $\div$  (conc. of potassium in water) is about 0.47 when 0.05 M KOH is shaken up with G.C. mixture, but the ratio (conc. bicarbonate in G. C. mixture)  $\div$  (conc. bicarbonate in *C*) is probably less than 0.001 when 0.063 M  $\text{KHCO}_3$  at pH 7.3 is shaken up with G.C. mixture (see p. 548). Hence relatively little  $\text{KHCO}_3$  will pass through *B*. During the outward passage of  $\text{KHCO}_3$  some of it will react to form KG and  $\text{CO}_2$  (and some of the latter will escape). When  $\text{KHCO}_3$  is allowed to diffuse outward and no  $\text{CO}_2$  is bubbled, some  $\text{KHCO}_3$  appears in *A* but there is some loss of  $\text{CO}_2$  (1) due to reaction with guaiacol and consequent escape into the air.

<sup>28</sup> Since the osmotic pressure in *A* is greater at the start than in *C* there may be a slight outward movement of water during the first portion of the experiment.

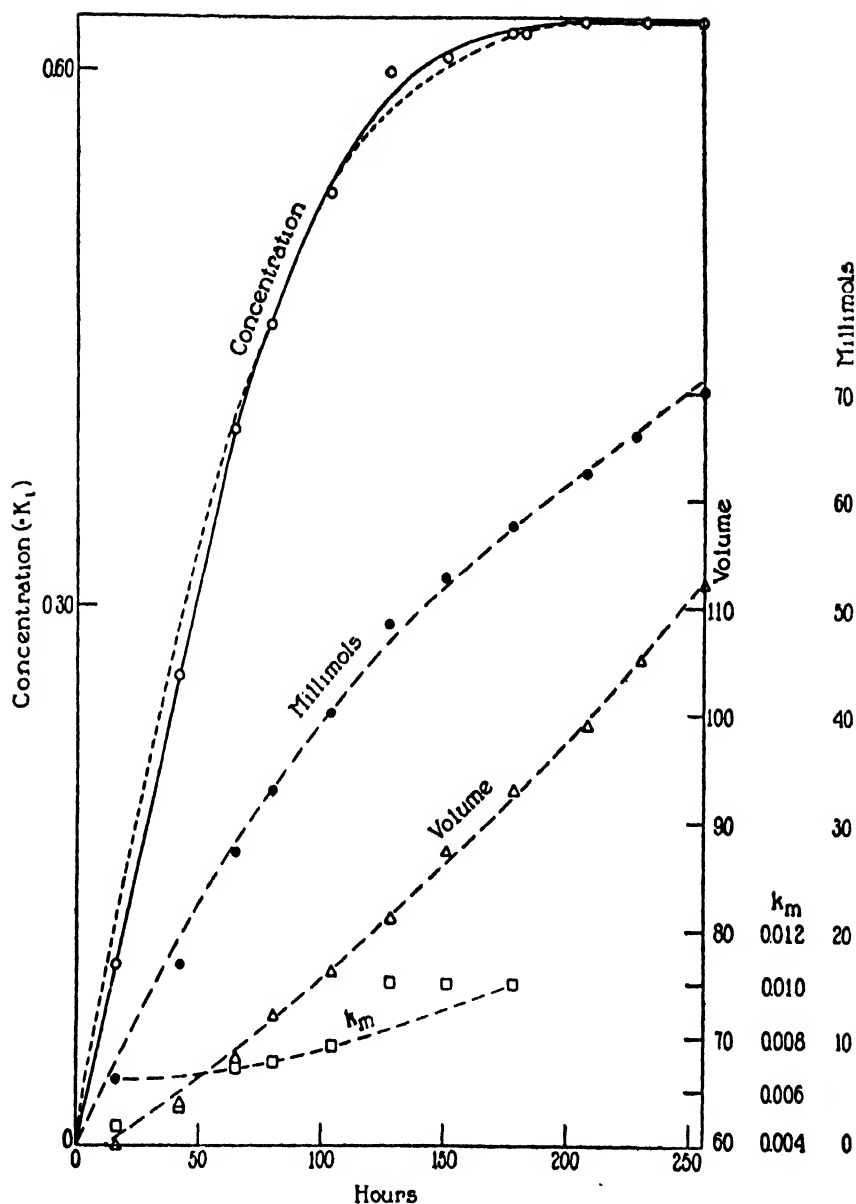


FIG. 3. Observed increase in  $K_i$  (unbroken line, drawn free-hand to give an approximate fit) and calculated values (broken line). Observed values of volume are represented by the symbol  $\Delta$ ; broken line shows values of  $58.1 e^{0.00288t}$ . Values of  $k_m$  calculated from observed values of  $K_i$  are represented by the symbol  $\square$ ; broken line shows values from calculated values of  $K_i$ . Millimols of observed values are shown by symbol  $\bullet$ ; broken line shows the curve obtained from calculated values of  $K_i$  and of volume.

curve of volume<sup>29</sup> agrees so well with the curve for 58.1  $e^{0.00259t}$  (broken line, Fig. 3) that we may put  $V_t = V_b e^{k_2 t}$ , where  $V_b$  is the calculated volume<sup>30</sup> of  $C$  at the beginning (58.1 cc.),  $V_t$  is the calculated volume at the time  $t$ , and  $k_2 = 0.00259$ .

At first the transfer of electrolyte is such that in unit time  $C$  gains more mols of electrolyte than liters of water and the concentration of electrolyte in  $C$  increases. But as time goes on the transfer of electrolyte decreases and that of water increases until a state is reached where in unit time  $C$  gains just as many mols of electrolyte as liters of water (Fig. 4). The concentration then becomes constant and the difference in osmotic pressure between  $A$  and  $C$  becomes sta-

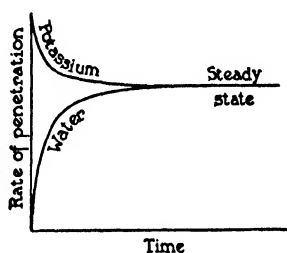


FIG. 4. Upper curve, rate of penetration of potassium (decreasing until the steady state is reached); lower curve, rate of penetration of water (increasing until the steady state is reached). Diagrammatic. The scale for potassium differs from that for water so that at the steady state if the rate for potassium were 0.63 mol per hour the rate for water would be 55.5 mols (1 liter) per hour.

tionary, so that the rate of water transfer should also become constant and the volume curve should become a straight line. This seems to be the case as far as can be judged by the experiments hitherto performed.

The exponential increase in volume will tend to make the concentration decrease exponentially. This may be expressed as follows.

If  $Q$  be the number of mols of potassium in  $C$  and  $V$  the volume we may put  $K_i = \frac{Q}{V}$ . What happens when  $Q$  is constant and water

<sup>29</sup> The figures for volume have been corrected for removal of samples. No correction was made for evaporation since in Model I (shown in Fig. 2 of the paper by Osterhout, W. J. V., and Stanley, W. M., *J. Gen. Physiol.*, 1931-32, **15**, 669) it is negligible.

<sup>30</sup> This differs but little from the observed value which is 60.

enters? At the time  $t_1$  we may call the values  $K_{i(1)}$  and  $V_{(1)}$  and at the time  $t_2$  we have  $K_{i(2)}$  and  $V_{(2)}$ . Hence  $K_{i(1)} = \frac{Q}{V_{(1)}}$  and  $K_{i(2)} = \frac{Q}{V_{(2)}}$ . But  $V_{(2)} = V_{(1)}e^{k_2(t_1 - t_2)}$ . Substituting this value we have  $K_{i(2)} = \frac{Q}{V_{(2)}} = \frac{Q}{V_{(1)}e^{k_2(t_1 - t_2)}} = \frac{Qe^{-k_2(t_1 - t_2)}}{V_{(1)}} = K_{i(1)}e^{-k_2(t_1 - t_2)}$ . Hence the loss of  $K_i$  due to the entrance of water is exponential and in any unit of time is equal to  $K_i k_2$ .

### *The Time Curve of $K_i$*

The increment of  $K_i$  in any unit of time, with no water entering, is equal to the increase in mols ( $Q$ ) divided by the volume ( $V$ ). If this increment of  $K_i$  be called  $\left(\frac{dK_i}{dt}\right)_v$ , we may write

$$\left(\frac{dK_i}{dt}\right)_v = \frac{1}{V} \frac{dQ}{dt}$$

As already stated (p. 537) we regard  $\frac{dQ}{dt}$  as proportional to  $K'_o - K'_i$ , hence, if we assume that during the first unit of time no water enters, we may write

$$\left(\frac{dK_i}{dt}\right)_v = \frac{1}{V_i} C(K'_o - K'_i)$$

where  $C$  is a "constant."<sup>31</sup>

If (the volume being constant)  $K'_i$  should increase in each unit of time by an amount proportional to  $K'_o - K'_i$  the value of  $K'_o - K'_i$  must decrease in each unit of time by an amount proportional to itself; *i.e.*, would fall off exponentially.

<sup>31</sup> The value of this "constant" will depend on the various diffusion constants, the thickness of the layers, the shape of  $B$  and the areas of its two surfaces, the rate of stirring, the rate of decomposition of  $KG$  in  $C$ , and other variables which will be discussed in a subsequent paper. It will also depend on the viscosity of  $B$  which increases as its content of  $KG$  increases. Hence it is not strictly speaking a constant but may be regarded as such for our present purpose.

Hence it appears that two processes determine the value of  $K_i$ , *i.e.*, a process which increases it is followed by one which decreases it: or

1. The value of  $K_i$  is increased by the entrance of potassium and should no water enter the rate might fall off in a manner approximately exponential.

2. As potassium penetrates and the osmotic pressure in  $C$  rises the resulting entrance of water causes a falling off in the value of  $K_i$  and this proceeds exponentially (p. 541).

Such a situation calls to mind the succession of events in a radioactive series, where a substance  $A$  breaks down to form  $B$ , at a rate which decreases exponentially, while  $B$  breaks down in the same way: and this suggests that it may be worth while to see how far a treatment involving successive exponential processes may fit the present case. A formula similar to that employed for a radioactive series may be written as follows:<sup>32</sup>

$$K_i = S_b \left( \frac{k_1}{k_2 - k_1} \right) (e^{-k_1 t} - e^{-k_2 t})$$

where  $S_b$ ,  $k_1$ , and  $k_2$  are constants (for the significance of these constants see p. 544). Despite certain differences between the kinetic situation here and that in a radioactive series this formula fits the observations fairly well, as is seen in Table I and Fig. 3.

One point of difference is that the first process (penetration of potassium) is influenced by the second (entrance of water). Among the consequences of this are the following:

1. If  $K_i$  increase more rapidly (*e.g.* as the result of more rapid stirring)  $K_i'$  will also increase more rapidly. Hence  $S$  will decrease more rapidly and  $k_1$  must increase: but  $k_2$  will do likewise since water will enter more rapidly. Any change therefore which multiplies the value of  $k_1$  tends to multiply that of  $k_2$  by the same factor. But if  $k_1$  and  $k_2$  be multiplied by the same factor the essential properties of the time curve will not change since all the abscissae will be divided by this

<sup>32</sup> Rutherford, E., *Radioactive substances and their radiations*, Cambridge, University Press, 1913. Mellor, J. W., *Chemical statics and dynamics*, London, 1909. Osterhout, W. J. V., *Injury, recovery, and death, in relation to conductivity and permeability*, Philadelphia, J. B. Lippincott Co., 1922. Thiersch, F., *Z. physik. Chem.*, 1924, **111**, 175.

same factor<sup>33</sup> (the value of the maximum will not change but the time required to reach the maximum will be divided by the factor mentioned).

2. When  $K_i$  becomes constant in the steady state, owing to the fact that water and electrolyte enter in a constant ratio,  $K_i'$  will also become constant and the value of  $K_o' - K_i'$  will no longer change.<sup>34</sup>

TABLE I

Concentration of Potassium ( $K_i$ ) and Millimols of Potassium in  $C$ , Volume ( $V$ ) of  $C$ , and "Monomolecular" Constant  $k_m$

Hours	Volume		$K_i$		Millimols of K in $C = K_i (V)$		$k_m = \frac{1}{t} \log \frac{K_s}{K_s - K_i}$	
	Obs.	Calc.	Obs.	Calc.	From obs. values	From calc. values of vol. and $K_i$	From obs. values	From calc. values
	cc.	cc.			millimols	millimols		
0	60	58	0	0	0	0	—	—
16	60	61	0.1	0.13	6.0	7.9	0.0047	0.0063
42	64	65	0.26	0.30	16.6	19.5	0.0055	0.0067
65	68	69	0.40	0.41	27.2	28.3	0.0068	0.0070
80	72	72	0.46	0.46	33.0	33.1	0.0071	0.0071
104	76	76	0.53	0.53	40.3	40.3	0.0077	0.0077
128	81	81	0.60	0.58	48.6	47.0	0.0100	0.0086
151	87	86	0.61	0.60	53.0	51.6	0.0100	0.0088
178	93	92	0.62	0.62	57.7	57.0	0.0100	0.0100
183	—	—	0.62	0.62	—	—		
208	99	99	0.63	0.63	62.4	62.4		
232	105	106	0.63	0.63*	66.2	66.8		
256	112	113	0.63	0.63*	70.6	71.2		

\* These values hold only if the steady state has been reached at which time the method of calculation changes.

<sup>33</sup> Cf. Osterhout, W. J. V., Injury, recovery, and death, in relation to conductivity and permeability, Philadelphia, J. B. Lippincott Co., 1922.

<sup>34</sup> In this respect the situation resembles that of a reversible monomolecular reaction, but the time curve differs since in the present case it is not of the first order. Cf. Mellor, J. W., Higher mathematics for students of chemistry and physics, London, Longmans, Green and Co., 1922, 228. Lewis, W. C. McC., A system of physical chemistry, London, Longmans, Green and Co., 1921. Since  $(K_o' - K_i')$  is constant  $\frac{dQ}{dt}$  is also constant.

3. The dependence of the first process on the second is taken into account in deriving the equation: the steps are as follows. Since  $K_i = Q + V$  we have

$$dK_i = \frac{VdQ - QdV}{V^2}$$

Substituting  $\frac{Q}{V} = K_i$  we have

$$dK_i = \frac{1}{V} dQ - \frac{K_i}{V} dV$$

Hence

$$\frac{dK_i}{dt} = \frac{1}{V} \frac{dQ}{dt} - \frac{K_i}{V} \frac{dV}{dt}$$

Substituting  $\frac{dQ}{dt} = C(K_o' - K_i')$  and  $\frac{dV}{dt} = Vk_2$  we have<sup>35</sup>

$$\frac{dK_i}{dt} = \frac{C(K_o' - K_i')}{V} - K_i k_2$$

We may now put  $C(K_o' - K_i') = S'$ : if this value were to fall off exponentially so that  $S' = S_b' e^{-k_0 t}$  (where the subscript  $b$  designates the value at the beginning and  $k_0$  is a constant) we should have

$$\frac{dK_i}{dt} = \frac{S_b' e^{-k_0 t}}{V} - K_i k_2$$

<sup>35</sup> As a numerical illustration we may put  $K_o' = 100$ ,  $K_i' = 10$ ,  $K_i = 10$ ,  $V = 100$ ,  $Q = 1000$ , and  $C_1 = 1$ . Then since  $\frac{\Delta Q}{\Delta t} = C_1(K_o' - K_i')$  we have for one unit of time  $\Delta Q = 100 - 10 = 90$ . During this unit of time let us suppose that  $V$  increases from 100 to 101 which makes  $k_2 = 0.01$ . We then have

$$\begin{aligned} \frac{\Delta K_i}{\Delta t} &= \frac{C(K_o' - K_i')}{V} - K_i k_2 \\ &= \frac{100 - 10}{100} - 10(0.01) \\ &= 0.8 \end{aligned}$$

This may be checked as follows. At the start  $K_i = \frac{Q}{V} = \frac{1000}{100} = 10$ . At the end of the unit of time  $K_i = \frac{1090}{101} = 10.8$ . Hence  $\frac{\Delta K_i}{\Delta t} = 0.8$ .

Substituting  $V = V_b e^{k_2 t}$  gives

$$\frac{dK_i}{dt} = \frac{S'_b e^{-k_0 t} e^{-k_2 t}}{V_b} - K_i k_2$$

Putting  $k_1 = k_0 + k_2$  and  $S_b = \frac{S'_b}{V_b(k_1)}$  gives

$$\frac{dK_i}{dt} = S_b e^{-k_1 t} k_1 - K_i k_2$$

This formula merely states that in any unit of time the increase in  $K_i$  equals that which would occur if no water entered during that unit of time (*i.e.*  $\frac{C(K_0' - K_i')}{V}$  which may under certain conditions be regarded as  $S_b e^{-k_1 t} k_1$ ) less the diminution due to the entrance of water during that unit of time (*i.e.*  $K_i k_2$ ).

A differential equation of this type is used to describe radioactive change and when integrated gives an equation of the form appearing on p. 542.

#### *Calculation of $k_1$*

We approximate  $k_1$  by observing how rapidly  $\frac{dQ}{dt}$  falls off with time and this approximation is corrected by trial.<sup>36</sup> In this way we arrive at the value  $k_1 = 0.0084$ .

#### *Calculation of $S_b$ , $K_s$ , and Time Required to Reach the Steady State*

Returning to the formula

$$\begin{aligned} \frac{dK_i}{dt} &= S_b e^{-k_1 t} k_1 - K_i k_2 \\ &= S_b e^{-k_0 t} e^{-k_2 t} (k_0 + k_2) - K_i k_2 \end{aligned}$$

we see that during the first hour, when no water is entering  $C$ , we may put  $k_2 = 0$ : hence during the first unit of time

$$\frac{dK_i}{dt} = S_b k_0$$

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<sup>36</sup> Dr. L. G. Longworth has devised a method based on that given by Running (*cf.* Running, T. R., Empirical formulas, New York, John Wiley and Sons, Inc., 1917) for getting the value of  $k_1$  graphically.



Now  $k_0 = k_1 - k_2 = 0.0084 - 0.00259 = 0.00581$  and the corrected value of  $\frac{\Delta K_i}{dt}$  at the start is 0.00616. Hence

$$0.00616 = S_b(0.00581)$$

$$S_b = 1.06$$

Having the values of  $S_b$ ,  $k_1$ , and  $k_2$  we may calculate the value of  $K_s$  in the steady state, which we may call  $K_s$ . We have<sup>32</sup>

$$\begin{aligned} K_s &= S_b \left( \frac{k_1}{k_2} \right)^{\left( \frac{k_2}{k_2 - k_1} \right)} \\ &= (1.06) \left( \frac{0.0084}{0.00259} \right)^{\left( \frac{0.00259}{0.00259 - 0.0084} \right)} \\ &= (1.06) (3.243)^{-0.44686} \\ &= 0.63 \end{aligned}$$

This agrees with the observed value of 0.63 (the value of  $K_s$  obtained in this way depends only on  $S_b$  and on the ratio  $k_1 \div k_2$  and not on the actual values of  $k_1$  and  $k_2$ ).

We may now calculate the time required to reach the steady state. Calling the value of  $S$  in the steady state  $S_s$ , we have<sup>32</sup>

$$\begin{aligned} S_s k_1 &= K_s k_2 \\ S_s &= S_0 e^{-k_1 t} = \frac{K_s k_2}{k_1} \\ e^{-k_1 t} &= \frac{K_s k_2}{S_b k_1} \\ e^{-k_1 t} &= \frac{(0.63)(0.00259)}{(1.06)(0.0084)} \\ e^{-0.0084 t} &= 0.183 \end{aligned}$$

We find from a table<sup>37</sup> that

$$e^{-1.697} = 0.183$$

hence

$$t = 202$$

This is within 2 per cent of the observed value of 208.

### *Calculation of $K_i$*

For the time curve we have<sup>38</sup>

$$\begin{aligned} K_i &= S_b \left( \frac{k_1}{k_2 - k_1} \right) (e^{-k_1 t} - e^{-k_2 t}) \\ &= (1.06) \left( \frac{0.0084}{0.00259 - 0.0084} \right) (e^{-k_1 t} - e^{-k_2 t}) \\ &= -1.53 (e^{-0.0084 t} - e^{-0.00259 t}) \end{aligned}$$

For example, at 80 hours we have

$$\begin{aligned} K_i &= (-1.53) (e^{-0.672} - e^{-0.2072}) \\ &= (-1.53)(0.5107 - 0.8129) \\ &= (-1.53)(-0.3022) \\ &= 0.46 \end{aligned}$$

which agrees with the observed value of 0.46. A comparison of the calculated and observed values (Table I and Fig. 3) shows fair agreement throughout.

<sup>37</sup> Cf. Becker, G. F., and Van Orstrand, C. E., Hyperbolic functions, Smithsonian mathematical tables, Washington, The Smithsonian Institution, 3rd reprint, No. 1871, 1924.

<sup>38</sup> The measurements of  $K_i$  and of volume might have been made more carefully had there been any intention of using them for mathematical analysis.

### Calculation of $k_m$

It is sometimes convenient to classify time curves according to their behavior with reference to the value of the apparent velocity constant  $k_m = \frac{1}{t} \log \frac{a}{a-x}$ . (For example, if we treat a bimolecular curve in this way we find that  $k_m$  falls off with time.<sup>39</sup>) In the present case we find that the value of  $k_m = \frac{1}{t} \log \frac{K_s}{K_s - K_i}$  (where  $K_s$  is the value of  $K_s$  in the steady state) increases with time as shown in Fig. 3 and Table I.

### Values of $K'_o$ and $K'_s$

Can we ascertain the values of  $K'_o$  and  $K'_s$ ? An attempt was made to approximate them as follows. On shaking up 0.04 M KG + 0.01 M KHCO<sub>3</sub> with G.C. mixture it was found that the ratio: (potassium in aqueous solution) ÷ (potassium in non-aqueous mixture) was 0.38 (most of the potassium in the non-aqueous mixture being KG, since the partition coefficient of KHCO<sub>3</sub> is apparently very low). Hence we may assume that in the present case, where *A* contains 0.041 M organic potassium salts and 0.009 M KHCO<sub>3</sub>, KG in the outer surface of *B*, would be about (0.05) 0.38 = 0.019 M.<sup>40</sup>

In order to ascertain the value of  $K'_s$ , 0.63 M KHCO<sub>3</sub> approximately saturated with CO<sub>2</sub> at atmospheric pressure (the pH being 7.3) was shaken with a small quantity of G.C. mixture. The concentration of potassium in the G.C. mixture was 0.0062. That of KHCO<sub>3</sub> in the G.C. mixture is not known but by analogy with KCl we may regard it as very small. Hence that of KG is probably not less than 0.0057.

It seems possible to get some idea of the value of  $K'_s$  in another way. We proceed as follows. We have seen earlier (p. 536) that we may write  $(KG)' = C_o C_s (K) (OH)$ , where  $(KG)'$  is the activity of undissociated KG in *B*. The concentration of KG in *B* may be regarded as equal to  $a'_{KG}$  divided by  $\gamma'$ , the activity coefficient in *B*. Hence if  $\gamma'$ ,  $C_o$ , and  $C_s$  happen to be about the same for  $K'_o$  and for  $K'_s$ ,

<sup>39</sup> Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, **13**, 695.

<sup>40</sup> Actually it would be less since there is a concentration gradient in the unstirred layer *A<sub>B</sub>* (Fig. 1).

(the concentration of undissociated KG in the inner surface of  $B_i$  in the steady state) we may put

$$\frac{(K_s)'}{(K_o)'} = \frac{(K_s)(OH_s)}{(K_o)(OH_o)}$$

where  $(K_s)$  and  $(OH_s)$  refer to activities in  $C$  in the steady state and  $(K_o)$  and  $(OH_o)$  to activities in  $A$ . The concentration of potassium outside is 0.05 M and inside 0.63 M: the pH value outside<sup>3</sup> is 9.1 (hence  $OH_o = 4.9$ ) and that inside is 7.6 (hence  $OH_s = 6.4$ ). Substituting these values, inserting the activity coefficients<sup>41</sup> and putting  $K'_o = 0.019$  we have

$$\begin{aligned} K'_s &= \frac{(0.63)(0.63)(10^{-6.4})}{(0.05)(0.817)(10^{-4.9})} \\ &= 0.31 \\ K'_s &= 0.0059 \end{aligned}$$

This figure is, of course, uncertain since we do not know the activity coefficients accurately.

#### *Relation of the Rate to the Value of $K'_o - K'_i$*

If, as stated earlier (p. 537), the rate of entrance of mols be proportional to  $K'_o - K'_i$ , we should be able to calculate the latter from the former. We proceed as follows. Taking the rate of entrance of mols at the beginning, which we may call  $R_b$ , as the average during the first measured period, we have  $R_b = 6.0 \div 16 = 0.375$  millimol per hour. The rate in the steady state (between 232 and 256 hours), which we may call  $R_s$ , is 0.183 millimol per hour. Hence  $R_s \div R_b = 0.183 \div 0.375 = 0.5$ .

Let us now consider whether we get the same value for the ratio  $(K'_o - K'_i \text{ in the steady state}) \div (K'_o - K'_i \text{ at the start})$ . Substituting the values previously ascertained we obtain for this ratio  $(0.019 - 0.0057) \div (0.019) = 0.7$ . The discrepancy between this

<sup>41</sup> 0.817 and 0.63 are activity coefficients. For explanation see preceding paper (1). Here as elsewhere we employ concentrations in place of activities for KG in  $B$ .

value and that of 0.5 derived from rates will be less when these values are corrected as follows.

1. The value of  $K_s$  (*i.e.* of  $K_i$  in the steady state) is undoubtedly too low since it was obtained with the external solution at pH 7.3. Increasing the pH to 7.6 (*i.e.* to the pH of the experiment) would increase the activity of the guaiacol ion (p. 535) and that of KG in *B*. If there were no complicating factors it would approximately double it (p. 535).

2. There is a factor which makes the ratio derived from rates appear too small since it causes the rate of entrance of KG in the steady state to appear less than it really is.

As the experiment progresses there is an increasing outward movement of  $\text{KHCO}_3$  (experimentally demonstrated elsewhere<sup>1</sup>). This reaches its maximum in the steady state and decreases the apparent rate of penetration of KG (what we actually observe is the inward rate of KG minus the outward rate of  $\text{KHCO}_3$ ).

3. An additional factor is that the value of  $K'_s$  is taken too low. In the shaking experiments the concentration of KG at the outer surface of *B* is 0.05 *M* giving 0.019 KG in *B*. But when we set up the model with 0.05 *M* KG in *A* the concentration at the outer surface of *B* is less because of the concentration gradient in the unstirred aqueous layer  $A_B$ . If we regard the actual concentration at the outer surface of *B* as low enough to make the value of  $K'_s$  0.0125 the ratio becomes  $(0.0125 - 0.0057) \div 0.0125 = 0.5$ .

When we correct for these factors the ratio derived from rates becomes larger and that derived from concentration gradients becomes smaller so that the discrepancy tends to disappear.

Even when these corrections are applied we should not expect the ratio derived from rates to be equal to that derived from concentrations, because the ratio derived from rates is lowered by factors which do not affect the other ratio in the same way. Such factors are the increase in the thickness of  $B_o$  and  $B_i$  and the decrease of the diffusion constants in these layers due to the increase in the viscosity<sup>42</sup> of the G.C. mixture which accompanies the increase in the concentration of KG. If these factors affect the rate of penetration of potassium

<sup>42</sup> That such an increase occurs is shown by experiments made in this laboratory by W. M. Stanley.

and of water to the same extent they will not change the value of  $K_i$  in the steady state and hence will not affect the value of  $K'_o - K'_i$ ; but they will diminish the value of  $R_i$ ,<sup>43</sup> and hence lower the value of  $R_i \div R_o$ .

In view of these considerations the agreement between the ratios derived from rates and that derived from concentration gradients seems to be fully as good as could be expected.

### *Exchange of Ions*

It is sometimes stated that in living cells the penetration of potassium depends on an exchange of  $K^+$  for  $H^+$ , the ions passing as such through the protoplasm. Can this conception be applied to the model?

When KOH is placed in *A* it reacts with HG to form KG which in turn reacts with  $CO_2$  in *C* to form  $KHCO_3$ . The net result is that *A* loses  $K^+$  and *C* loses  $H^+$  which is equivalent thermodynamically to an exchange of  $K^+$  for  $H^+$  (though the actual transport through *B* may be mostly in molecular form, *e.g.* as undissociated HG and KG).

As stated in the preceding paper<sup>1</sup> it seems probable that the concentration of ions in the G.C. mixture (whose dielectric constant is low) is small compared with that of molecules and unless the mobility of the ions were extremely high ionic exchange would play a subordinate rôle. The following calculations bear this out.

From considerations set forth elsewhere<sup>44</sup> an exchange of  $K_o^+$  for  $H_i^+$  might be expected to proceed according to the formula  $\frac{dQ}{dt} = C_7 [(H_i)(K_o) - (K_i)(H_o)]$ , where  $C_7$  is a constant.

We may use the following values for activities.<sup>1</sup> At the start:<sup>41</sup>  $(K_o) = (0.05)(0.817) = 0.04$ ,  $(H_o) = 10^{-9.1}$ , and  $(K_i) = 0$ . The value of  $(H_i)$  at the start, which we may call  $(H_s)$ , will depend on the rate at which  $CO_2$  is bubbled but it may be estimated tentatively as  $10^{-5.3}$ . At the steady state we have<sup>41</sup>  $(K_o) = 0.04$ ,  $(H_o) = 10^{-9.1}$ ,  $(K_i) = (0.63)(0.63) = 0.4$ , and  $H_i = 10^{7.6}$  (this will be called  $H_s$ ).

We have seen (p. 537) that when  $\frac{dQ}{dt}$  is regarded as proportional to

<sup>43</sup> To judge from the literature this diminution may be very considerable (16).

<sup>44</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1930-31, **14**, 277.

$(K_o)(OH_o) - (K_i)(OH_i)$ , calling  $\frac{dQ}{dt}$  in the steady state  $R_s$  and  $\frac{dQ}{dt}$  at the start  $R_b$ , we have (p. 549)

$$\begin{aligned}\frac{R_s}{R_b} &= \frac{(K'_o - K'_i)}{K'_o} = \frac{(K_o)(OH_o) - (K_i)(OH_i)}{(K_o)(OH_o)} \\ &= \frac{(0.05)(0.817)(10^{-4.9}) - (0.63)(0.63)(10^{-6.4})}{(0.05)(0.817)(10^{-4.9})} = 0.7\end{aligned}$$

But if the penetration of potassium proceed by ionic exchange and if  $\frac{dQ}{dt}$  must be regarded as proportional to  $(H_i)(K_o) - (K_i)(H_o)$  we must put

$$\begin{aligned}\frac{R_s}{R_b} &= \frac{(H_i)(K_o) - (K_i)(H_o)}{(H_o)(K_o)} \\ &= \frac{(10^{-7.6})(0.04) - (0.4)(10^{-9.1})}{(10^{-5.3})(0.04)} \\ &= 0.003\end{aligned}$$

This is widely divergent from the observed value of 0.5 (p. 549) for the observed ratio between the rates of entrance of mols at the steady state and at the start.

If ionic exchange proceeded in this way it would make little or no difference whether the K ions in *A* were accompanied by guaiacol ions or by chloride ions, but we find experimentally that KG penetrates very much faster than KCl.

But it might be said that the ionic exchange is in reality between the outer surface of  $B_o$  and the inner surface of  $B_i$ . In order to estimate the value on this basis we may proceed as follows. Since the activity coefficient of KG in the G.C. mixture cannot be far from unity we may for comparative purposes regard it as unity and employ concentrations. The activity of  $K^+$  in the outer surface of  $B_o$  may be called  $(K_o^+)'$  and the other ions may receive similar designations. We then have in the outer surface of  $B_o$

$$(K_o^+)' (G_o^-)' = C_{KG} K'_o$$

where  $C_{KG}$  is the dissociation constant of  $KG$  in the G.C. mixture. Then we may write as an approximation

$$\begin{aligned}(K_o^+)' &= \sqrt{C_{KG}} \sqrt{K_o'} \\ &= \sqrt{C_{KG}} \sqrt{0.019} \\ &= \sqrt{C_{KG}} (0.138)\end{aligned}$$

Taking the concentration of  $KG + KHCO_3$  in the inner surface of  $B_i$  to be 0.0062 (as stated on p. 548) we may for purposes of calculation lump them together and call both  $KG$  (this would introduce no serious error if the dissociation constants in  $B$  were similar as is probably the case). The activity of  $K^+$  in the inner surface of  $B_i$  in the steady state may be called  $(K_s^+)'$ . Hence we may put

$$\begin{aligned}(K_s^+)' &= \sqrt{C_{KG}} \sqrt{0.0062} \\ &= \sqrt{C_{KG}} (0.079)\end{aligned}$$

As we do not know the activities of the hydrogen ion in the surfaces of  $B$  (which are supposed to be in approximate equilibrium<sup>17</sup> with the adjoining aqueous phases) we may assume for purposes of calculation that they are equal to the activities in  $A$  and in  $C$  multiplied by a constant  $C_b$ . The activity of the hydrogen ion in the outer surface of  $B_o$  may be called  $(H_o^+)'$ ; that in the inner surface of  $B_i$  at the start  $(H_b^+)'$  and in the steady state  $(H_s^+)'$ . This gives  $(H_b^+)' = C_b 10^{-5.3}$ ,  $(H_o^+)' = C_b 10^{-9.1}$ , and  $(H_s^+)' = C_b 10^{-7.6}$ . Substituting these values in the equation  $\frac{R_s}{R_b} = \frac{(H_s^+)'(K_o^+)' - (K_s^+)'(H_o^+)' }{(H_b^+)'(K_o^+)'}$  we have

$$\begin{aligned}\frac{R_s}{R_b} &= \frac{C_b(10^{-7.6}) \sqrt{C_{KG}}(0.132) - \sqrt{C_{KG}}(0.079)C_b(10^{-9.1})}{C_b(10^{-5.3}) \sqrt{C_{KG}}(0.138)} \\ &= \frac{(10^{-7.6})(0.132) - (0.079)(10^{-9.1})}{(10^{-5.3})(0.138)} \\ &= 0.005\end{aligned}$$

The values for (rate at steady state)  $\div$  (rate at start) obtained from calculations based on ionic exchange (*i.e.* 0.003 and 0.005) diverge



widely from that obtained by direct observation of the number of mols passing through at the start and at the steady state; *i.e.*, 0.5 (see p. 549). The value 0.5 is in much better agreement with that obtained by assuming that potassium moves through *B* in the form of molecules of KG. This value is 0.7 (p. 549) which is known to be too high since it takes no account of such factors as those mentioned on p. 550.

#### CONCLUDING REMARKS

The calculations indicate that exchange of ions; *i.e.*, of  $K^+$  in *A* for  $H^+$  in *C*, does not play an essential rôle. Hence it would seem that potassium must move through *B* chiefly in the form of undissociated KG or as the ion pair  $(K^+)' + (G^-)'$ . As the concentration of ions is presumably small, owing to the very low dielectric constant<sup>45</sup> of the G. C. mixture, they have been neglected in order to simplify the calculations. This would seem to be permissible in the present case in view of the result of the calculations. But it is, of course, inaccurate and becomes increasingly so the more dilute the solutions employed.

Ionization in *B* would not change the form of the equations, but the rate would become a different function of  $(K)$   $(OH)$ . For example if the dissociation in *B* were practically complete we might have<sup>46</sup>

$$\begin{aligned} \frac{R_s}{R_b} &= \frac{\sqrt{(K_o)(OH_o)} - \sqrt{(K_s)(OH_s)}}{\sqrt{(K_o)(OH_o)}} \\ &= \frac{\sqrt{(0.05)(0.817)(10^{-4.9})} - \sqrt{(0.63)(0.63)(10^{-4.4})}}{\sqrt{(0.05)(0.817)(10^{-4.9})}} \\ &= 0.4 \end{aligned}$$

<sup>45</sup> This is probably in the neighborhood of 11.

<sup>46</sup> If we have nearly complete dissociation in *A* and *B*, a constant activity partition coefficient ( $S_0$ ) for the undissociated potassium guaiacolate, and  $(K^+)'$  =  $(G^-)'$  in *B*, the activity of undissociated potassium guaiacolate in *A* may be designated as  $(KG)$  and that in the outer surface of  $B_o$  as  $(KG)'$ . We may put  $(K_o)(G_o) = k(KG)$ ; also  $(KG)' = S_0(KG)$  and  $(KG)' = k'(K^+)'(G^-)'$ , where  $k$  and  $k'$  are the dissociation constants of  $(KG)$  and  $(KG)'$ . Then  $(K^+)'(G^-)' = k'(KG)' = k'(S_0)(KG) = k'(S_0)(K_o)(G_o) \div k$ . Putting  $k'(S_0) \div k = H$  (*i.e.* a constant) we have  $(K^+)'(G^-)' = H(K_o)(G_o)$  and  $(K^+) = \sqrt{H(K_o)(G_o)}$ .

This agrees better with the observed ratio (0.5) than does the calculated value (0.7) given on page 549. On this basis therefore it would seem quite possible (with the rather dilute solutions here employed) to have some dissociation in *B*. But the calculations involve assumptions and uncertainties (*e.g.* in regard to the ionic activity coefficients). This will be discussed in later papers.<sup>47</sup>

The equation for the time curve may be regarded as empirical. It implies that when no water enters the rate of entrance of potassium may fall off in a manner approximately exponential and that the entrance of water tends to produce an exponential decrease in concentration. Hence the kinetics may be treated as that of consecutive exponential processes. This is, of course, only an approximation but it may serve to prepare the ground for a more rigorous treatment.

At the start the observed time curves of concentration and of volume lag behind the calculated. This is to be expected since the calculated curve predicts that some potassium will enter *C* during the first second but as a matter of fact it may take much longer for the first molecules of the potassium compounds to cross the non-aqueous layer and enter *C*. The thicker the non-aqueous layer, the more constricted in any region, and the slower the rate of stirring, the greater is the lag, so that the time curve of concentration might under some conditions become convex toward the base line at the start. In

When the concentration in *B*<sub>0</sub> is small we may use concentrations in place of activities and say that the concentration of diffusing ion pairs (*i.e.* of  $(K^+)' + (G^-)'$ ) is equal to  $(K^+)'$  which is proportional to  $\sqrt{(K_0)(G_0)}$  and hence (page 536) to  $\sqrt{(K_0)(OH_0)}$ .

<sup>47</sup> The partition coefficient would be affected. If the concentration of potassium guaiacolate in *B* divided by that in *A* be called *S* it is evident that the value of *S* will depend on the degree of dissociation in *A* and *B*. If we put (46)  $S_0 = (KG)' \div (KG)$  we have the following relations for dilute solutions. With no dissociation in *B* we have  $S \div S_0 = 1 - \alpha$ , where  $1 - \alpha$  is the fraction of undissociated molecules in *A*. With dissociation in *B* it can be shown that  $\frac{S}{S_0} = (1 - \alpha)$

$\left(1 + \sqrt{\frac{k'}{CS_0(1 - \alpha)}}\right)$  where *C* is the concentration in *A*. This enables us to estimate the value of *k'*. This will be discussed in later papers.

view of this and other considerations we may expect considerable variation in the time curve and this seems to be the case as far as can be judged from rough measurements made in some other experiments.<sup>48</sup>

The situation in the model may well be analogous to that in *Valonia* and *Nitella*. The protoplasm appears to consist of two non-aqueous layers corresponding to  $B_o$  and  $B_i$ , but having an aqueous layer between them. The aqueous solutions corresponding to  $A$  and  $C$  are the external medium and the sap, both of which are well stirred by convection currents.<sup>49</sup> The cell wall, of course, corresponds to an unstirred layer.

*Note.*—After the acceptance of this article the attention of the author was called to a paper by Brooks and Brooks<sup>50</sup> which deals with the kinetics of models. In their paper diffusion constants and gradients are ignored on the ground that there are no unstirred layers. Such a condition appears, however, to be unattainable in practice.<sup>16</sup>

#### SUMMARY

An organic potassium salt,  $KG$ , passes from an aqueous phase,  $A$ , through a non-aqueous layer,  $B$ , into a watery solution,  $C$ . In  $C$  it reacts with  $CO_2$  to form  $KHCO_3$ . The ionic activity product ( $K$ ) ( $G$ ) in  $C$  is thus kept at such a low level that  $KG$  continues to diffuse into  $C$  after the concentration of potassium becomes greater in  $C$  than in  $A$ . Hence potassium accumulates in  $C$ , the osmotic pressure rises,

<sup>48</sup> By varying the values of  $k_1$  and  $k_2$  a variety of curves can be obtained, some showing a progressive increase, others a decrease, and still others approximate constancy of  $k_m$  (cf. Osterhout, W. J. V., Injury, recovery and death, in relation to conductivity and permeability, Philadelphia, J. B. Lippincott Co., 1922). In the last case the equations for a monomolecular reaction would apply.

In a series of experiments with models we find indications that all of these occur (depending on rate of stirring, etc.) although the measurements are not precise enough to be used for mathematical analysis.

<sup>49</sup> The extent of such stirring is often underestimated. See footnote 16.

<sup>50</sup> Brooks, S. C., and Brooks, M. M., *J. Cellular and Comp. Physiol.*, 1932, **2**, 53.

and water goes in. A steady state is eventually reached in which potassium and water enter *C* in a constant ratio.

The rate of entrance of potassium (with no water penetrating into *C*) may fall off in a manner approximately exponential. But water enters and may produce an exponential decrease in concentration. This suggests that the kinetics may be treated like that of two consecutive monomolecular reactions. Calculations made on this basis agree very well with the observed values.

The rate of penetration appears to be proportional to the concentration gradient of *KG* in the non-aqueous layer and in consequence depends upon the partition coefficients which determine this gradient.

Exchange of ions (passing as such through the non-aqueous layer) does not seem to play an important rôle in the entrance of potassium.

The kinetics of the model may be similar to that of living cells.



## ULTRAFILTRATION

### II. "BOUND" WATER (HYDRATION) OF BIOLOGICAL COLLOIDS\*†

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#### INTRODUCTION

At the present time there exists a very wide divergence of opinion regarding the state of the water in biological fluids and in solutions of the lyophilic colloids. On the one hand it is held that the colloidal material in these solutions is hydrated to a high degree and that the water "bound" by the colloids in this way differs in solvent properties from water in bulk. This point of view is well illustrated by the following quotation taken from a recent review by Gortner (1):

"... more and more it is becoming evident that the water in living tissues and even in inanimate colloidal systems does not exist wholly as "free" water such as characterizes water in bulk, . . . a greater or smaller fraction of the water is intimately "bound" to the organic structures and becomes an essential part of the disperse phase as contrasted with the free water of the dispersion medium."

The opposite point of view is that all but a small fraction of the water in the biological and colloidal systems is in its usual solvent state. Thus, A. V. Hill (2), Grollman (3), and Sunderman (4) have, in recent investigations, come to the conclusion that the "free" water of blood, blood corpuscles, muscle, egg white, and various other protein solutions is nearly equal to the total water content in these systems. The status of the subject is rendered even more unsatisfactory by the wide divergence in the values of the "bound" water found in the same systems by the use of the different means that have been suggested for

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estimating the state of the water. Concretely to illustrate this, we have collected in Table I the published figures for the "bound" water in gelatin solutions as determined by the various methods. Gelatin was chosen for this illustrative table because it has been extensively studied. Many other colloidal systems might equally well have been selected. The table gives the method used, the content of gelatin,

TABLE I

*Published Values for the "Bound" Water in Gelatin Solutions as Determined by Various Methods*

Method used	Gelatin content	Gm. of "bound" water per gm. of gelatin	Authors
	<i>per cent</i>	<i>gm.</i>	
Cryoscopic with sucrose as reference substance	1-5	2.0 at lowest gelatin content to 1.0 at highest	Newton and Martin (5)
Freezing out method; H <sub>2</sub> O measured calorimetrically		2.0	Thoenes (6)
Freezing out method; H <sub>2</sub> O measured with dilatometer	2-32	4.7 at lowest gelatin content to 0.7 at highest	Jones and Gortner (7)
Vapor pressure method			
Reference substance sucrose	1	3.0	Grollman (3)
Reference substance KCl	1	3.0	Grollman (3)
Reference substance NaCl	1	1.0	Grollman (3)
Freezing out method; analysis of gelatin residue	12-40	0.53	Moran (8)
Contraction in volume		0.08	Svedberg (9)
Osmotic pressure deviation from van't Hoff's law	1-14	4.7	Burk and Greenberg (10)
Viscosity	1-14	7 at lowest gelatin content to 3.35 at highest	Kunitz (11)

the value found for "bound" water per gram of gelatin, and the authors who carried out the investigation. From the table it is seen, and this is equally true for other systems extensively investigated, that there is no agreement whatsoever on the amount of water "bound" by the gelatin in solution.

The disagreement in part is no doubt due to the different physical properties that are measured by the various methods, but there is no

agreement even with the same procedure. It is seen in Table I, for example, that Thoenes, Jones and Gortner, and Moran each report different values of the "bound" water by the freezing out method. Hill states that various methods of defining "free" water are possible and that the different definitions do not necessarily coincide with each other. He defines the "free" water fraction as the weight of water in 1 gm. of fluid or tissue which can dissolve substances added to it with a normal depression of the vapor pressure. Any definition of "free" water, it is readily seen, is inherent in the physical nature of the particular experimental procedure employed and the statement that various ways of defining "free" water are possible simply signifies that the different experimental methods which have been devised for determining the state of the water do not measure the same intrinsic physical property.

Since all the colligative properties of a solution are thermodynamically interrelated, and with sufficient data are calculable one from the other, Hill's definition can be made more general by changing it to state that the weight of water in 1 gm. of fluid or tissue which can dissolve substances added to it with a normal change in colligative properties is the "free" water. The "bound" water, it follows, is the water in the system which has lost its normal solvent properties.

On this reasoning, many of the procedures that have been used for determining the state of the water in biological and colloidal systems are identical in principle and should give the same results for the fractions of "free" and "bound" water present. It seems strange then that in the hands of one group of investigators, methods based on the same thermodynamic principles should indicate high values of "bound" water in systems in which another group of investigators find practically no "bound" water. The answer to the difficulty here would seem to lie in the correct interpretation of the experimental results obtained. That this interpretation is not above question is indicated by the fact that the significance of the results of the two chief methods in use, the freezing point lowering method of Newton and Gortner (12) and the vapor pressure lowering method of Hill (2), have recently been the subject of dispute.

Grollman (3) thinks that Newton and Gortner did not apply a sufficient correction for the water taken up for hydration by the sucrose



used as the reference substance by these authors, while Sunderman (4) and Briggs (13) conclude that no correction whatsoever should be made for the hydration of the sucrose. Hill's method comes under question in two ways. In this method there is actually measured the temperature difference between the unknown and the reference solution due to an evaporation of water from one and a condensation on the other. This temperature difference, it is assumed, is proportional to the vapor pressure difference between the two solutions. It is possible that this proportionality may not always hold with some of the complex systems investigated. Another objection against Hill's figures is based on Briggs' reasoning (13) that the absolute amount of water associated with a colloid ("bound" water) varies with the activity coefficient of the water with which it is in equilibrium and is not a constant fraction of the total water. Since the activity coefficients of the water in the systems studied by Hill were not considered, the values of the "bound" water estimated were lower than the true amounts. The reader is referred to Briggs' paper for the complete argument, which is too involved and extensive to be given in detail here.

On reading over the literature on the subject, the impression becomes strong that the controversy is due largely to a failure in some quarters to appreciate the wide deviation in colligative properties of the systems under consideration from those of an ideal solution. The "bound" water hypothesis attempts to account for the departure from the ideal solution laws in systems containing colloids in a manner analogous to the hypothesis of hydrates in solution which, at a little earlier period, had considerable vogue in explaining the deviation of crystalloid solutions from ideal solution laws (14).

This is simply and illuminatingly illustrated by the directly measured osmotic pressures of both protein solutions and certain crystalloids such as sucrose. As has been pointed out, among others by Burk and Greenberg (10), the osmotic pressure of even isoelectric protein solution departs from van't Hoff's law starting with very dilute solutions and with crystalloids such as sucrose at higher molal concentrations. The departure from van't Hoff's law both for the proteins and the crystalloids has been attributed to the formation of hydrates with the solute and thus a loss of solvent properties by a portion of the water present. This explanation was favored by Burk

and Greenberg. But it is equally plausible, and more in harmony with the results to be given below, that the departure from van't Hoff's law is due to other causes for the deviation from the ideal solution laws than the formation of hydrates.

For these reasons, an independent method for determining the state of water, free from the difficulties of interpretation of the procedures based on the measurement of colligative properties of solutions, would be of great value in testing the "bound" water hypothesis. With colloidal solutions, such as are here our concern, properly carried out ultrafiltration experiments offer such a method.

#### EXPERIMENTAL

The generally accepted criterion of "bound" or hydrate water is that it has lost its normal solvent properties. Accordingly, if a crystalloidal reference substance is added to a colloidal solution it should distribute itself only in the "free" water of the solution. If a portion of the solution is ultrafiltered through a membrane which permits only the passage of the solvent and other crystalloids, holding back the colloidal constituents, the concentration of the reference substance in the ultrafiltrate liquor becomes a measure of the "free" water in the colloidal solution provided certain criteria given below are met. The calculation of the "bound" water from such an experiment may be made as follows. Let  $C_T$  be the concentration of the reference substance per 1 gm. of total water in the system,  $P$  the amount of the colloid per gm. of total water, and  $h$  the "bound" water per gm. of colloid. Then the concentration of the reference substance with respect to "free" water is given by

$$C_u = \frac{C_T}{1 - hP}.$$

$C_u$ , from what has been said, is the concentration per gm. of water of the reference substance in the ultrafiltrated liquid. From this the bound water per gm. of colloid in the solution is given by

$$h = \frac{1}{P} \left( 1 - \frac{C_T}{C_u} \right)$$

The conditions that may invalidate this method are that a part of the reference material reacts with or is adsorbed by the colloid. In

such an event the reference substance would filter out in a lower concentration than its value present in the original solution. The other difficulty that may arise is one pointed out in the first publication of this series (15). If an electrolyte is used as the reference material when the colloid carries an electric charge, the ultrafiltration, as was there shown, becomes analogous to a Donnan membrane distribution and the conditions necessary for evaluating the state of the water fail. From this it follows that a non-electrolyte must be employed as the reference compound except in systems in which the colloidal constituents are uncharged.

The method proposed here for determining the state of the water was suggested by McBain and Jenkins (16), but their experiments were invalidated by the use of salts as reference substances with soap solutions. Recently, McBain and Kistler (17) have employed this method to determine the hydration of sucrose in solution. An analogous method, in which the reference substance is allowed to reach a diffusion equilibrium and the distribution is then determined, has been suggested by Weber and Nachmannsohn (18), Oda (19), and Eggleton (20).

A study of the state of the water in a number of systems containing biologically important colloids has been carried out by the ultrafiltration method outlined here. The general ultrafiltration procedure was the same as that described in a previous publication (15). The two reference substances generally employed were urea and glucose, but a number of salts were also used for this purpose with isoelectric gelatin solutions. The solutions in these experiments, excepting blood sera, were made up by first preparing the crystalloidal constituents in the desired concentration, a portion was set aside for the analysis of the reference substance and to another measured volume the desired amount of the dry colloid was added. The colloidal solution was then subjected to ultrafiltration and the ultrafiltrated liquor was analyzed for the reference substance. In this way, all the analyses were carried out on a basis of equivalent volumes. The Van Slyke gasometric method was used for the urea analysis (21), and Hanes' (22) modification of the Hagedorn-Jensen method for glucose. The determination of the salts in the ultrafiltrate experiments with isoelectric gelatin was made by conductivity measurement.

The data of the experiments are given in Tables II, III, and IV for solutions of gelatin, casein, and miscellaneous other colloids including blood sera. The column headings in the tables give the content of the colloid, the crystalloidal composition of the solvent, the reference

TABLE II

*Test for "Bound Water" of Gelatin Solutions by Ultrafiltration*

Gelatin concentration	Solvent composition	Reference substance	Reference substance in original solution	Reference substance in the protein-free ultrafiltrate
<i>per cent</i>			<i>gm. per liter</i>	<i>gm. per liter</i>
1.65	0.01 N HCl	Urea	1.09	1.09
2.20	0.025 N HCl	Urea	2.00	2.05
2.67	0.10 N HCl	Urea	1.29	1.20
2.54	0.075 N HCl	Urea	1.40	1.25
2.74	0.26 N HCl	Urea	1.38	1.38
3.00	0.011 N NaOH	Urea	0.99	0.95
3.70	0.005 N KCl	Urea	1.13	1.138
2.90	0.01 N NaCl	Urea	2.11	2.05
2.73	0.0075 N KCl	Urea	1.57	1.60
3.00	H <sub>2</sub> O	Urea	1.415	1.39
3.00	H <sub>2</sub> O	Glucose	1.00	0.99
3.38	H <sub>2</sub> O	Glucose	1.00	1.008
3.11	H <sub>2</sub> O	Glucose	1.00	1.00
2.63	0.1 N NaCl	Glucose	1.00	0.994
3.16	0.0043 N HCl	Glucose	1.158	1.159
4.85	0.100 N KCl	KCl	0.100*	0.0995*
5.00	0.100 N KCl	KCl	0.100*	0.1000*
4.80	0.0500 N KCl	KCl	0.0500*	0.0490*
3.00	0.0246 N NaCl	NaCl	0.0246*	0.0245*
3.12	0.0465 N Na <sub>2</sub> SO <sub>4</sub>	Na <sub>2</sub> SO <sub>4</sub>	0.0465*	0.0463*
5.00	H <sub>2</sub> O	Urea	9.87	9.80†
4.00	0.005 N NaOH	Urea	9.87	9.84†
4.00	0.091 N HCl + 0.08 N NaCl	Urea	26.70	26.60†
5.00	0.1 N KCl	Urea	26.70	26.80†

\* Concentration in gram equivalents per liter.

† Urea determined by Kjeldahl method for nitrogen.

compound used, and the analytical figures for the reference substance in the original solution and in the ultrafiltrate. Mostly small concentrations of reference substance were used, purposely, so that the objection could not be raised that in some way the amount of reference

substance upset the usual state of the water in the colloidal solutions. The use of urea as a reference substance has been deprecated because it manifests abnormal colligative properties, but since the measurements depend merely on the analysis of the total amount of urea, the objection can have no weight here.

TABLE III  
*Test for "Bound Water" of Casein Solutions by Ultrafiltration*

Casein concentration	Solvent composition	Reference substance	Reference substance in original solution	Reference substance in the protein-free ultrafiltrate
<i>per cent</i>			<i>gm. per liter</i>	<i>gm. per liter</i>
3.25	0.023 N NaOH	Urea	1.435	1.425
2.28	0.05 N KOH + 0.01 N KSCN	Urea	1.45	1.45
1.75	0.01 N KOH + 0.02 N $\text{KC}_2\text{H}_3\text{O}_2$	Urea	0.78	0.79
1.18	0.0067 N KOH + 0.03 N $\text{KC}_2\text{H}_3\text{O}_2$	Urea	0.78	0.78
3.46	0.030 N KOH + 0.019 N $\text{K}_2\text{C}_2\text{O}_4$	Urea	0.61	0.60
1.44	0.012 N KOH + 0.0145 N $\text{K}_2\text{C}_2\text{O}_4$	Urea	0.57	0.59
2.66	0.0145 N NaOH	Glucose	0.979	0.986
2.94	0.0154 N NaOH	Glucose	0.882	0.882
2.27	0.0119 N NaOH	Glucose	0.909	0.907

If it is accepted that "bound" water loses its usual solvent properties, the figures given in the tables convincingly lead to the conclusion that within the limits of error of the methods of analysis there is no detectable amount of "bound" water in solutions of gelatin, casein, blood sera, glycogen, or starch, dissolved in the solvent mixtures here employed. Here and there an individual experiment may give a different impression, but these are to be set down to analytical errors. One

way in which the presence of "bound" water in these experiments might have been concealed is that an adsorption of the reference compound by the colloid takes place in just exactly the right amount to balance the increase in its concentration in the ultrafiltrate due to the "bound" water. But it seems inconceivable that such would be the case with all the colloidal materials, and with the different reference substances of these experiments. Particularly should it be noted that in the gelatin solutions no indication of the presence of consider-

TABLE IV

*Test for "Bound Water" of Miscellaneous Colloidal Solutions*

Colloidal solution	Reference substance	Reference substance in original solution	Reference substance in the ultrafiltrate
		gm. per liter	gm. per liter
Blood serum (dog) . . . . .	Urea	0.590	0.590
Blood serum (beef) . . . . .	Urea	0.440	0.420
Blood serum (beef) . . . . .	Urea	0.287	0.292
Blood serum (pig) . . . . .	Urea	0.260	0.270
Blood serum (beef) . . . . .	Glucose	1.16	1.15
Blood serum (beef) . . . . .	Glucose	1.14	1.13
5 per cent soluble starch solution . . . . .	Urea	2.06	2.075
6.5 per cent corn starch solution . . . . .	Urea	1.63	1.63
1.0 per cent pectin solution . . . . .	Urea	2.20	2.10
5.0 per cent glycogen solution . . . . .	Urea	1.224	1.220
4.0 per cent glycogen solution . . . . .	Urea	1.224	1.218

able amounts of "bound" water is given by five separate compounds used as reference substances.

The experimental results given reduce the amount of water possibly "bound" by these colloids to a small fraction of a gm. of water per gram of colloid or less. We do not wish to deny that water in these small amounts may be associated with the proteins and the starch and glycogen examined, but the presence of the huge values of "bound" water up to several grams of water to each gram of protein, as listed in Table I for gelatin, is in our eyes not tenable.

These experiments have an important significance for the current theories of the stability of colloidal solutions. The lyophilic colloids, which include most of the biologically important colloids, are largely

stable in solution even in an uncharged condition. This stability has been attributed to these colloids being highly hydrated so that the films of the hydrate water prevent the colloids from coalescing when they collide due to the Brownian movement, and thus keep them from flocculating out of the solution. This viewpoint has been most prominently cultivated by Kruyt (23) and his coworkers. With the failure of the hypothesis of a high hydration, it becomes also necessary to revise the theory of stability based upon it. We would propose in its place an extension of the Langmuir-Harkins theory of molecular orientation. Significant in this connection is the following statement taken from a paper published by Langmuir in 1925 (24): "It is reasonable to assume that the field of force about any particular group or radical in a large organic molecule is characteristic of that group and, as a first approximation, is independent of the nature of the rest of the molecule." It seems plausible then that the nature of the force fields of particular groups and the number of these groups in a lyophilic colloid determine its stability in solution. A preponderance of polar groups would favor dispersion in a polar solvent such as water. Measures that augmented the force fields of the polar groups would increase the stability; *e.g.*, ionization. On the other hand, a decrease in the force fields would decrease the stability. The reagents which are assumed to produce flocculation by dehydration probably act in this way.

#### SUMMARY

Assuming that "bound" water loses its solvent properties, it is shown by an ultrafiltration method that in solutions of gelatin, casein, starch, and glycogen, and in blood serum, only a very small fraction of the water can be associated with the colloids in this form.

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## SIMILARITY OF THE KINETICS OF INVERTASE ACTION IN VIVO AND IN VITRO. III

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It has been pointed out in the initial paper of this series (1) that in the hydrolysis of sucrose by the enzyme, invertase, the course of the reaction is identical both when invertase solutions and when living yeast cells are used as the source of inverting activity. A second comparison (2) of the kinetics of invertase action *in vivo* and *in vitro*, in which the pH-activity relationship of the enzyme was studied under these two conditions, showed that the activity of this hydrolytic enzyme was affected by changes in the  $[H^+]$  of the reaction medium in exactly the same manner and to the same extent both when the enzyme used had been extracted from the yeast cells by autolysis and when the living yeast cells themselves were used as the source of the inverting action. As a result of this identity it was suggested that the sucroclastic action of live yeast cells is located in the outer region of the cells where their invertase content would be most freely exposed to the effect of changes in the pH of the suspending medium. In an effort to obtain more evidence to support this suggestion it was decided to investigate a third kinetic relationship, the effect of changing sucrose concentration upon the hydrolytic activity of yeast cells and of invertase solutions obtained therefrom.

Of the many investigations of the effect of the concentration of sucrose upon the velocity of its hydrolysis by means of invertase solutions, the first complete one is that of Ingersoll (3) who found that the velocity of hydrolysis increases with increasing sucrose concentration, reaching a maximum at about 5 per cent, beyond which the velocity steadily decreases. It was subsequently shown by Nelson and Schubert (4) that this decrease in velocity is caused largely by the diminution of the water concentration as the sucrose concentra-

tion increases; thus, the concentration of water as well as of the sucrose is a factor which influences the kinetics of invertase action.

In this work the effect of changing the sucrose concentration over the range of from 2 to 25 per cent upon the velocity of sucrose inversion by living yeast cells and by invertase solutions prepared from them is determined and compared.

#### EXPERIMENTAL

##### *Effect of Varying Sucrose Concentrations upon the Hydrolytic Activity of Invertase Solutions*

The invertase preparation used was prepared by autolyzing *Saccharomyces cerevisiae* with toluene and water. The invertase was precipitated from the autolysate in 50 per cent ethyl alcohol and extracted from this precipitate with water. In determining the velocities of hydrolysis 25 cc. of the enzyme solution were pipetted into 100 cc. of sucrose solutions of such concentrations as to give final sucrose concentrations of 2, 5, 10, 15, 20, and 25 per cent. The hydrolyzing solutions contained 0.01 M acetate buffer, pH 4.5, and were kept at  $25^{\circ} \pm 0.01^{\circ}\text{C}$ . during the reaction. At recorded time intervals 25 cc. samples were removed and to each was added a drop of sodium hydroxide solution of sufficient strength to raise the pH to 8–9, at which pH hydrolysis ceases. After allowing time for mutarotation to occur the samples were polarized at  $25^{\circ}\text{C}$ . in a 2 dm. tube using a mercury arc light of wave length  $546.1\text{ }\mu\mu$ .

The results of these experiments are presented in Table I.

##### *Effect of Varying Sucrose Concentrations upon the Hydrolytic Activity of Living Yeast Cells*

The inverting agent in these experiments was a suspension of washed, pressed *S. cerevisiae*. 25 cc. of this suspension were pipetted into 100 cc. samples of sucrose solutions of such concentrations as to give final sucrose concentrations of 2, 5, 10, 15, 20, and 25 per cent. The hydrolyzing mixtures containing 0.01 M acetate buffer at pH 4.5 were shaken continuously in a thermostat at  $25^{\circ} \pm 0.01^{\circ}\text{C}$ . 25 cc. samples were removed and inversion stopped as described above and were immediately filtered cell-free through porous bottom Gooch crucibles. The filtrates were polarized as in the preceding experiments.

The results of these experiments are presented in Table II, *a* and *b*.

The results tabulated in Tables I and II are represented graphically by Fig. I in which the velocity of hydrolysis is plotted against sucrose concentration.

TABLE I

*Variation of Hydrolytic Activity of Invertase Solutions with Sucrose Concentration*

Sucrose concentration per 100 cc. of solution	$\Delta T$	Rotation	$\Delta$ Rotation	Change per min. (mean value)
gm.	min.	degrees	degrees	degrees
2	0	3.13		
	6	2.80	0.33	
	12	2.47	0.66	0.0548
	18	2.15	0.98	
5	0	7.86		
	6	7.45	0.41	
	12	7.06	0.80	0.0674
	18	6.65	1.21	
10	0	15.64		
	10	14.99	0.65	
	20	14.36	1.28	0.0644
	30	13.71	1.93	
15	0	23.43		
	10	22.84	0.59	
	20	22.23	1.20	0.0597
	30	21.63	1.80	
20	0	31.23		
	10	30.70	0.53	
	20	30.13	1.10	0.0541
	30	29.60	1.63	
25	0	39.03		
	10	38.55	0.48	
	20	38.03	1.00	0.0492
	30	37.54	1.49	

The velocity-sucrose concentration curves obtained by using yeast cells are practically identical with that obtained by using a solution of invertase obtained from the same yeast and all three curves are in agreement with those of Ingersoll (3) and of Nelson and Schubert (4)

in that, after reaching a maximum at about 5 per cent, the velocity gradually falls off as the sucrose concentration is increased. Since Nelson and Schubert have shown that the shape of this curve is the

TABLE II, a

*Variation of Hydrolytic Activity of Yeast Suspensions with Sucrose Concentration*

Sucrose concentration per 100 cc. of solution	$\Delta T$	Rotation	$\Delta$ Rotation	Change per min. (mean value)
gm.	min.	degrees	degrees	degrees
2	0	3.13		
	7	2.67	0.46	
	17	2.01	1.12	0.0658
	21	1.75	1.38	
5	0	7.85		
	7	7.30	0.55	
	17	6.51	1.34	0.0783
	27	5.76	2.09	
10	0	15.63		
	7	15.10	0.53	
	17	14.34	1.29	0.0760
	27	13.57	2.06	
15	0	23.47		
	7	22.97	0.50	
	17	22.26	1.21	0.0710
	27	21.57	1.90	
20	0	31.31		
	7	30.85	0.46	
	17	30.20	1.11	0.0655
	27	29.54	1.77	
25	0	39.15		
	7	38.73	0.42	
	17	38.11	1.04	0.0608
	27	37.50	1.65	

result of the two varying factors, sucrose concentration and water concentration, this identity of results *in vivo* and *in vitro* indicates that the invertase of the yeast cell is as freely exposed to the conditions of sucrose and water concentrations which prevail in its suspending

medium, and is just as sensitive to changes in these two variables as if it were contained in a cell-free solution.

The results of the investigation of this third kinetic relationship are entirely in agreement with the suggestion made in the second paper

TABLE II, *b*

Sucrose concentration per 100 cc. of solution	$\Delta T$	Rotation	$\Delta$ Rotation	Change per min. (mean value)
<i>gm.</i>	<i>min.</i>	<i>degrees</i>	<i>degrees</i>	<i>degrees</i>
2	0	3.15		
	6	2.77	0.38	
	11	2.44	0.71	0.0641
	16	2.12	1.03	
5	0	7.83		
	7	7.29	0.54	
	17	6.53	1.30	0.0769
	27	5.75	2.08	
10	0	15.64		
	7	15.11	0.53	
	17	14.36	1.28	0.0755
	27	13.60	2.04	
15	0	23.45		
	7	22.96	0.49	
	17	22.27	1.18	0.0697
	27	21.57	1.88	
20	0	31.33		
	7	30.88	0.45	
	17	30.24	1.09	0.0641
	27	29.61	1.72	
25	0	39.11		
	7	38.69	0.42	0.0595
	17	38.11	1.00	
	27	37.50	1.61	

of this series (2) to the effect that the invertase of yeast cells exerts its physiological activity neither in the cell sap nor in the cytoplasmic layer surrounding it, but in a region closer to the external surface of the cell where the effect of changing environmental conditions, either

hydrogen ion or sucrose and water concentrations, would be most pronounced.

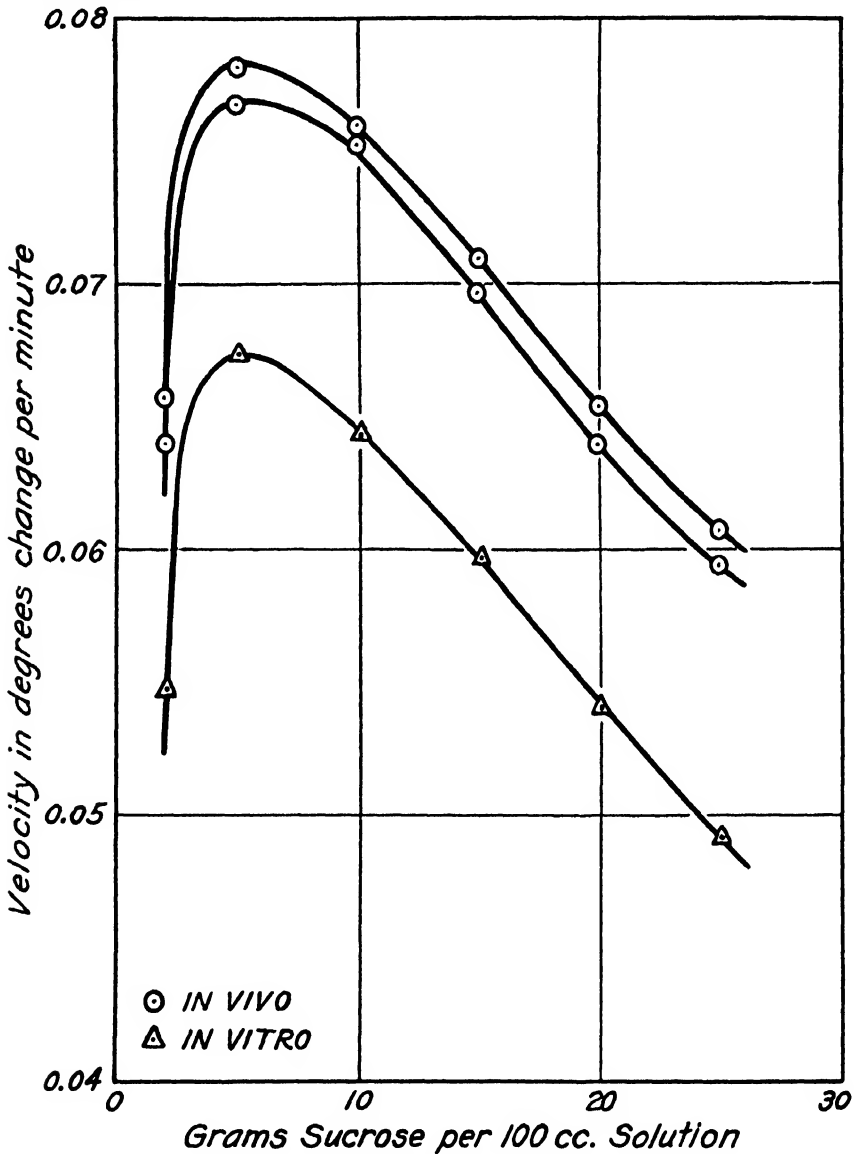


FIG. 1

The proof that the yeast cells used in this work were living after being suspended in a 25 per cent sucrose solution for 30 minutes was obtained by using the procedure employed in the preceding article (2).

## SUMMARY

1. The relationship of sucrose and water concentration to invertase activity *in vivo* and *in vitro* has been studied under the same environmental conditions.

2. The sucroclastic activity of *S. cerevisiae* cells and of invertase solutions prepared from them reacts to changes in sucrose and water concentration in an identical manner.

3. The invertase contained in living yeast cells is just as freely exposed to the conditions of sucrose and water concentrations of the suspending medium as it would be if it were contained in a cell-free solution. Weight is added to the previous suggestion (2) that yeast invertase exerts its physiological activity in a region quite close to the surface of the cell.

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# AN EXPERIMENTAL COMPARISON OF DIFFERENT CRITERIA OF DEATH IN YEAST\*

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Definitions of death are mostly negative. We call an organism dead when it shows no more the symptoms which we consider characteristic of life. Staining with certain dyes, which is frequently used as a distinction between living and dead cells, is also a negative criterion, being caused by the loss of selective permeability of the plasma membrane. The standard definition of bacteriologists considers a bacterium dead if it does not produce colonies on agar; the criterion is loss of reproductive power.

To establish a basis for comparison, the authors compared the death rates of a pure culture yeast as measured by various criteria. The yeast was a bottom-fermenting beer yeast, grown on raisin extract + 0.5 per cent yeast extract + 0.5 per cent  $\text{KH}_2\text{PO}_4$ . After 2 days' growth at  $30^\circ$ , the yeast was centrifuged from its medium and kept near  $0^\circ$  in a buffer solution of pH 4.6.

The criteria observed were: (1) loss of reproduction, measured by the plate count on raisin agar; (2) loss of fermentation, measured by the  $\text{CO}_2$  pressure from 3 cc. of yeast suspension in glucose, by means of a fermentometer (Rahn, 1929); (3) change of staining reaction, measured by mixing equal amounts of yeast suspension and 0.01 per cent methylene blue solution; (4) loss of selective permeability, measured by mixing equal parts of yeast suspension and 0.2 per cent Congo red solution. Cells which are stained by the last two methods are considered to be dead. In addition to this, the coagulation of the protoplasm was observed occasionally, in the dark-field (Bancroft and Richter, 1931) as well as in the light-field.

\* This investigation has been made possible through a grant by the Heckscher Research Foundation.

Four causes of death were tested: (a) high temperature, near 50°C.; (b) chemical poisoning, represented by  $\text{HgCl}_2$ ; (c) ultraviolet light,

TABLE I

*Survivors of Yeast Cells, under Various Conditions, Measured by Different Criteria*

Time of exposure	Plate count, cells per cc.	Mercury pressure in 6 min. from 3 cc. of yeast suspension	Percentage of cells not stained by	
			Methylene blue	Congo red

Exposed to 0.025 per cent $\text{HgCl}_2$				
min.		mm.Hg	per cent	per cent
0	382,000,000	21.6	100	100
2.5	170,500,000	6.75	83.4	82.6
5	86,900,000	3.40	64.8	58.4
10	31,600,000	2.40	34.8	47.8
20	8,500,000	1.20	13.6	26.4
40	130,000	—	7.9	9.4

Exposed to 50°C.				
0	264,000,000	18.00	100	100
2.5	253,000,000	10.50	94.3	93.0
5	234,800,000	7.00	87.2	85.9
10	46,700,000	6.00	82.9	82.4
20	1,100,000	3.00	75.7	71.8
40	10,000	0.20	20.0	17.7

Exposed to ultraviolet light				
0	489,500,000	31.3	100	
2.5	302,500,000	25.5	102.9	
5	390,500,000	21.3	103.3	
10	135,300,000	20.0	100.6	
20	16,200,000	19.0	99.5	
40	500,000	13.8	97.0	

Exposed to x-rays				
0	630,000,000	35.6	100	
2.5	545,000,000	36.0	102.1	
5	445,000,000	34.0	109.8	
10	282,000,000	30.7	107.3	
20	170,000,000	24.0	106.2	
40	145,000,000	24.0	100.9	

from a mercury vapor lamp, unfiltered; (d) x-rays, from two Coolidge tubes, one operated at 80 kv., 4 ma., with the yeast about 10 cm. from

the molybdenum target, and the other with 100 kv., 15 ma., with the yeast 16 to 23 cm. from the tungsten target.

TABLE II  
*Percentage of Survivors as Measured by Various Definitions*

Time of exposure	Plate count	CO <sub>2</sub> pressure	Cells not stained by methylene blue
Exposed to 50.8°C.			
<i>min.</i>			
0	100	100	100
2.5	83.5	90.2	90.1
5	20.3	35.2	44.5
10	0.0002	5.1	39.6
20	0.000006	2.5	19.5
40	0	0	0
Exposed to 0.025 per cent HgCl <sub>2</sub>			
0	100	100	100
2.5	44.5	31.2	83.5
5	22.7	15.7	64.8
10	8.3	11.1	34.8
20	2.3	5.5	13.6
40	0.3	—	7.9
Exposed to ultraviolet light			
0	100	100	100
2.5	69.7	79.0	103.4
5	57.6	61.3	100
10	27.4	60.0	94.2
20	1.8	60.0	94.5
40	0.7	39.0	85.8
Exposed to x-rays			
0	100	100	100
2.5	96.4	94.5	98.6
5	95.0	97.8	99.3
10	79.0	97.8	99.3
20	76.0	98.7	97.9
40	21.4	78.4	95.8

In each experiment, the survivors, as measured by the various criteria, were determined after 2.5, 5, 10, 20, and 40 minutes' expo-

sure to the harmful influence. In the case of  $\text{HgCl}_2$ , 1 cc. each of 0.1 molar potassium sulfide and 0.5 per cent ammonium chloride were given to 4 cc. of yeast suspension at these intervals, as antidote.

Samples of the results obtained by this method of procedure are shown in Table I.

From these data, the percentages of survivors for each criterion of death were computed. Table II shows the kind of data obtained. They show that with the four causes of death, the different criteria do not appear simultaneously. Only between the two dyes, no essential difference was observed, and they will be treated here as identical criteria. The decrease in the power of reproduction is always much larger than that of fermentation, and this again is larger than the decrease in resistance to dyes. There was practically no difference between the two dyes tested, methylene blue and Congo red, though it is supposed by some authors that the first might indicate loss of the reducing power of the protoplasm, while the other indicates the permeability of the plasma membrane.

In drawing conclusions from these data, it must be remembered that we are dealing with averages, and not with individual cells. We observe that more cells lose the reproductive power than the capacity to ferment. It is only an assumption, however, if we state that a cell first loses its faculty to grow, and then the faculty of fermentation, and third, the faculty of selective permeability. It has not been shown that only those cells which have lost the power to make colonies, lose their fermentative powers, and that this must precede the staining with dyes. There is some evidence for part of this sequence, but no proof. Since we have no means of separating "living" from "dead" cells, this proof could be brought only by observing the succession of criteria in isolated cells. This seems not entirely impossible, but very difficult to do.

For each criterion in each experiment, the death rate can be computed. Plotting the logarithms of the percentages of survivors against time, the resulting survivor curves are either straight lines or "sagging" curves. Rarely were bulging curves obtained.

Basically, the formula for the death rate with straight, and even with sagging, survivor curves (Rahn, 1930) is

$$K = \frac{1}{t} (\log a - \log b)$$

where  $a$  is the initial number of cells and  $b$  the survivors after the time  $t$ . Calculating the survivors in percentages of the initial number, we have

$$K = \frac{1}{t} (2 - \log b)$$

For different criteria of death in the same experiment, the formula would be

$$K' = \frac{1}{t} (2 - \log b')$$

$$K'' = \frac{1}{t} (2 - \log b'')$$

The ratio of these two rates which indicates how much faster the one symptom appears than the other, is

$$\frac{K'}{K''} = \frac{2 - \log b'}{2 - \log b''}$$

Instead of computing these ratios, they were obtained graphically from the smoothed logarithmic survivor curves where  $2 - \log b$  can be measured directly and accurately. The ratio is correct only when the survivor curves are rectilinear. When they are all sagging, the ratio is fairly accurate, for the sagging indicates that the more sensitive individuals are affected essentially in the same way. In the few cases where the curves for one criterion go one way and for another criterion in a different way, no reliable comparison is possible; these cases have been omitted.

The ratios are given in the next table. In order to allow direct comparison and averaging of the data, the ratios are all computed so that the loss of fermentation is 1 in each case.

### *Discussion of Results*

The averages of the death rate ratios appear as the most interesting result of this investigation. There is a tendency in these averages of the plate count ratio to increase with time. In the case of death by ultraviolet light, this increase is so large, and at the same time, a cor-

responding decrease of the methylene blue ratio is so conspicuous that this cause of death will be treated separately.

In averaging all the data of death caused by heat and by bichloride of mercury, the ratio of death as indicated by methylene blue, by

TABLE III  
*Ratios of Death Rates*

	Methylene blue staining				CO <sub>2</sub>	Loss of reproduction			
Death by heat									
	Exp. XIII	Exp. XIV	Exp. XV	Exp. XVI		Exp. XIII	Exp. XIV	Exp. XV	Exp. XVI
<i>min.</i>									
2.5	1.00	0.14	0.14	1.63	1	1.60	0.09	1.08	1.75
5	0.56	0.16	0.15	0.77	1	1.30	0.14	1.57	0.46
10	0.31	0.14	0.10	0.52	1	3.10	1.34	1.81	2.58
20	0.41	0.15	0.17	0.51	1	3.24	1.80	3.06	1.66
40	—	0.66	—	0.54	1	—	4.31	—	—
Averages									
<i>min.</i>									
2.5					0.73	1	1.13		
5					0.41	1	0.88		
10					0.27	1	2.21		
20					0.31	1	2.44		
40					0.60	1	—		
Death by HgCl <sub>2</sub>									
	Exp. XXI	Exp. XXIV	Exp. XXV	Exp. XXVI		Exp. XXI	Exp. XXIV	Exp. XXV	Exp. XXVI
<i>min.</i>									
2.5	0.26	0.38	0.15	0.09	1	1.30	1.90	0.69	0.75
5	0.13	0.53	0.24	0.36	1	1.18	1.30	0.80	0.71
10	0.26	0.56	0.46	0.83	1	1.78	1.95	1.08	1.33
20	0.27	0.96	0.70	0.83	1	2.34	2.05	1.30	1.46
40	0.37	—	—	—	1	2.50	—	—	—
Averages									
<i>min.</i>									
2.5					0.22	1	1.16		
5					0.32	1	1.00		
10					0.53	1	1.54		
20					0.69	1	1.79		

TABLE III—*Concluded*

	Methylene blue staining				CO <sub>2</sub>	Loss of reproduction			
Death by ultraviolet light									
	Exp. XVII	Exp. XVIII	Exp. XIX	Exp. XX		Exp. XVII	Exp. XVIII	Exp. XIX	Exp. XX
<i>min.</i>									
2.5	0.25	Not determined	0.36	—	1	0.75	1.56	2.27	1.91
5	0.11		0.05	—	1	—	0.67	0.91	0.63
10	0.05		0.19	(∞)	1	2.26	3.04	2.16	2.16
20	0.14		0.03	(∞)	1	1.54	3.82	5.81	5.04
40	0.07		0.02	(0.03)	1	2.94	4.95	5.61	8.34
	Averages								
<i>min.</i>									
2.5			0.30	1	1.62				
5			0.08	1	0.74				
10			0.12	1	2.41				
20			0.09	1	4.05				
40			0.05	1	5.46				
Death by x-rays									
						Exp. XXX	Exp. XXXI	Exp. XXXVI	Exp. XXXVIII
<i>min.</i>									
2.5				1	3.5	2.4	10.0	20.0	
5				1	3.9	7.9	8.5	20.0	
10				1	4.3	8.1	8.5	16.4	
20				1	4.4	7.9	6.3	12.2	
40				1	3.5	8.3	5.8	9.0	
	Averages								
<i>min.</i>									
2.5				1	9.9				
5				1	10.1				
10				1	9.3				
20				1	7.7				
40				1	6.6				

fermentation, and by plate count, is 0.46:1:1.52, or, in round numbers 1:2:3.

With x-rays, death as indicated by methylene blue or Congo red was too slow to be measured. Even the fermentation was but slightly



reduced, and the ratio of fermentation to plate count was very large, averaging 8.5. This difference is important. It indicates that loss of reproduction is not the immediate cause of the loss of fermentation. If so, the ratio should be approximately the same with all causes of death.

In death by ultraviolet light, the ratios do not remain constant, but increase with the time of exposure. While at first, after 2.5 minutes, the ratios of death by the three symptoms are 0.3 : 1:1.16, or roughly 1:3:4, they increase after 40 minutes to roughly 1:20:110. Death as indicated by methylene blue is very slow, and it seems that only a few weak cells are killed rather early while the rest are too resistant to succumb to these rays. But the ratio  $\frac{\text{Fermentation}}{\text{Plate count}}$  is also increasing

with time. The survivor curve for the plate count is nearly rectilinear, while that for fermentation is distinctly sagging. This appears strange because we are dealing with the inactivation of an enzyme; if this were a multimolecular process, the curve should bend in the opposite direction. The curve suggests that the enzyme molecules are not all equally resistant to ultraviolet light; some are more easily inactivated than others.

Loss of reproduction may be caused by a chemical change of one gene. It may or may not involve the repair mechanism of the cell. If the cell has lost the faculty of substituting new molecules for those used up in endogenous catabolism, the fermenting capacity must be expected to decrease, but this decrease is very slow (Rubner, 1913), even more so than that observed under the influence of x-rays. This would suggest that the loss of fermenting capacity as observed in these experiments must be largely due to a direct action of the harmful agent upon the enzyme itself. It should be remembered, however, that the zymases are for the most part not liberated, but combined with some part of the protoplasm, and that death of the cell means inactivation of this portion of the zymase (Rubner, 1913; von Euler and Lindner, 1915). Since the type of the compounds to which the zymases are anchored and their biological significance are not known, nothing further can be concluded from the material at hand.

The tendency for the ratio of loss of reproduction to loss of fermentation to become larger with time indicates that the survivor curves

of the two processes are not both strictly rectilinear. Either the slower process has a slightly sagging curve, or the faster process a slightly bulging curve.

The loss of selective permeability is considerably slower than the loss of fermenting capacity. The ratio fluctuates greatly because the error in the determination of survivors is quite large. The ratio is approximately the same with death by heat and by chemical poisoning. With x-rays, there was no death at all outside the limits of error when methylene blue was used as criterion, and very little death with ultraviolet light.

It is not possible to decide from these experiments whether loss of fermentation is the prerequisite for dye penetration. If some energy should be required to keep the cell membrane semipermeable, loss of fermenting capacity may mean loss of all energy, and therefore loss of semipermeability. It is just as likely, however, that the heat or poison acts upon the cell membrane as well as on zymases and chromosomes, and ultimately brings about loss of selective permeability independent of the changes in the inner cell mechanism.

The last criterion tested was the coagulation of the protoplasm which has been claimed by Bancroft and Richter (1931) to be the fundamental reaction of death in disinfection experiments. The coagulation of yeast plasma under harmful influences, as seen in the dark-field, is a very gradual process. The normal yeast cells contained ordinarily only one or two reflecting granules. The number of these granules increased under adverse conditions, and the decision whether a cell should be called coagulated or not is entirely arbitrary.

A yeast suspension was mixed with an  $\text{HgCl}_2$  solution so as to contain 0.025 per cent  $\text{HgCl}_2$ . A drop of this mixture was placed in the dark-field, and the number of "living" and "dead" cells counted every minute, with continuous shifting of the field, and with occasional renewal of the drop from the same suspension. After 2.5, 5, 10, 20, and 40 minutes, samples of the same suspension were shaken with the antidote mixture, and were investigated as usual. The result of one such experiment is given in Table IV and Fig. 1.

Coagulation of the protoplasm is the last evidence of death to appear, even though the authors considered the yeast cells coagulated long before they became as opaque as shown in the microphotographs by

TABLE IV  
*Percentages of Survivors as Measured by Various Criteria*

Time of exposure	Coagulation	Methylene blue	Plate	Time of exposure	Coagulation
Start				min.	
min.	83.5	71.4	100.0	22	75
2	76			23	65
2.5	—	31.6	0.7	24	49
3	76			25	42
4	83			26	69
5	62	23.7	0	27	59
6	76			28	30
7	80			29	26
8	76			30	22
9	67			31	11
10	67	4.8	0	32	24
13	80			33	8
14	65			34	8
15	62			35	17
16	70			36	9
17	70			37	9
18	76			38	5
19	67			39	1
20	66	0	0	40	0
21	43				

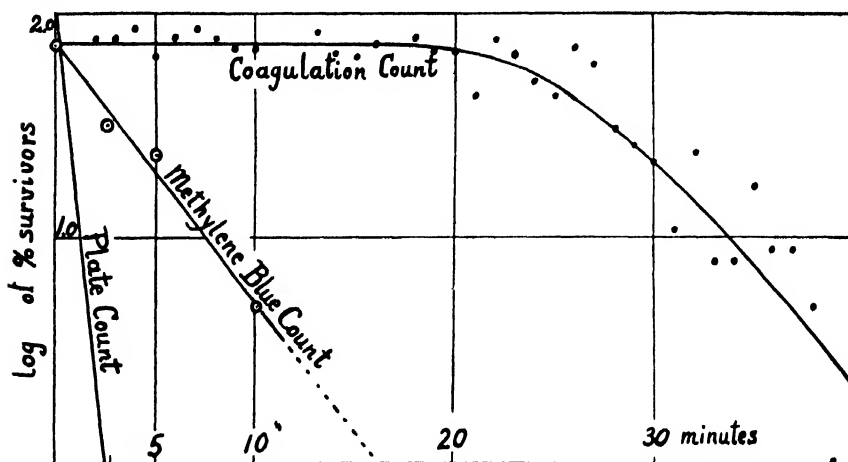


FIG. 1. Logarithmic survivor curves for the same yeast in 0.025 per cent  $\text{HgCl}_2$  measured by different criteria.

Bancroft and Richter. There is a long period of no deaths, and the logarithmic survivor curve is very bulging, which makes an accurate calculation of the death rate impossible. Using the datum of the 30 minute period for the coagulation rate, the following ratios are obtained:

Coagulation : methylene blue : plate count = 1:6:43.

It is very improbable from these facts that coagulation of the protoplasm is the real cause of death; *i.e.*, the fundamental reaction. Coagulation should not be considered as an explanation of disinfection, because the term disinfection is standardized as the loss of reproductive power of microorganisms, and this loss occurs forty times as fast as coagulation.

Coagulation in the light-field can be observed soon after coagulation in the dark-field. No quantitative measurements of this criterion have been made.

The senior author has advanced the theory that the logarithmic order of death of bacteria, which contrasts strikingly with the order of death of higher organisms, may be brought about by the fact that the destruction or inactivation of one single molecule (*e.g.* in one certain gene) prevents further cell division. The destruction of enzyme molecules by heat is probably a monomolecular reaction (Tammann, 1895), and a rectilinear logarithmic "survivor curve" may be expected. Loss of semipermeability, however, can hardly be a monomolecular process, and if semipermeability were destroyed purely according to chemical reaction, a bulging "survivor curve" should be expected. However, all experiments show rectilinear or sagging curves.

### *The Recovery Problem*

This problem is an entirely different one with unicellular and multicellular organisms. In the latter case, recovery may be accomplished if the uninjured cells possess sufficient vitality to reproduce, and thus to replace the inactivated cells by active ones. It may even be that the remaining uninjured cells are sufficient to carry out their specific function for the entire organism.

With unicellular beings, the simplicity of the cell mechanism makes recovery from injury more difficult; the simple multiplication of un-

injured parts is not possible. One healthy cell can develop into two cells, but one enzyme molecule cannot develop into two such molecules. Recovery of unicellular organisms means the reconstruction of a lost function.

Boycott (1920) raised the question whether bacteria can recover at all, considering the simple chemical appearance of their order of death. The very slow development of bacteria on plate cultures after injury (see *e.g.* Eijkman, 1908), leaves no doubt that generally speaking, single cells can recover from injury. For an analysis of this recovery, it is essential to consider the criteria used for death and injury.

We have no means of testing for a temporary loss of reproduction with bacteria. This test is possible only in sexual reproduction. The

TABLE V

*Percentage of Dead Cells as Measured by Methylene Blue Count*

	Length of exposure to HgCl <sub>2</sub>					
	0 min.	2.5 min.	5 min.	10 min.	20 min.	40 min.
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Examined immediately after exposure . . . . .	0	44.8	61.8	72.0	75.4	82.7
2 hrs., 25 min. later . . . . .	0	67.2	75.0	81.1	85.6	87.5

observation of greatly delayed growth need not necessarily imply injury of the growth mechanism, but might be explained through injuries of the energy-furnishing mechanism. It is generally assumed by cytologists and geneticists that a gene once destroyed can never be replaced by the cell. The cell either loses the power to reproduce, or if it reproduces, all the offspring lack this one gene and therefore differ from the original strain. It is not probable that microorganisms should make an exception to this rule. The growth mechanism consists of many other parts besides the genes, and these may be also very sensitive, but replaceable as long as the genes are intact.

Recovery of the fermenting capacity of the cells seems very probable. It has been shown by Buchner, Buchner, and Hahn (1903) that the zymase content of yeast cells may be increased by storing the compressed yeast on ice. Zymases must have been produced by the cells

once, and it is imperative that this faculty continue at least as long as the cell is capable of reproduction.

In most of our experiments, the fermenting capacity of the injured yeast was tested within 30 minutes after exposure, and again 2 to 4 hours later. There was a decided increase in the rate of fermentation during this time, but this was also the case with the controls, as one of the authors (Rahn, 1929) has shown before. The reason for this increase has not been explained satisfactorily as yet, unless it be due to production of more zymase by old cells in fresh sugar solution. For this reason, a recovery from injury cannot be considered proved.

The same test was applied to the methylene blue counts. If there were recovery, the percentage of "dead" cells immediately after exposure should be larger than a couple of hours later. The experiment did not verify this (Table V).

Bancroft and Richter mention (page 516) that the phenomenon of protoplasmic coagulation is reversible with yeast, without, however, giving records of the numbers of cells tested or proving that the "recovered" cells were really living. We have not repeated these experiments.

#### SUMMARY

Different criteria of death have been compared experimentally and quantitatively. Pure cultures of a yeast have been subjected to adverse conditions, and the number of dead cells, judged by different tests, has been determined in successive time intervals. The yeast cultures were exposed to heat, to  $\text{HgCl}_2$ , to ultraviolet light, and to x-rays.

In each case, the cells lost the power of reproduction (measured by the plate count) most rapidly. The loss of fermentation (measured by the  $\text{CO}_2$  pressure) was less rapid. Still slower was the change in staining reaction with methylene blue, and the loss of selective permeability of the plasma membrane (measured by the percentage of cells staining with Congo red). Slowest of all was the coagulation of protoplasm as observed in the dark-field.

In the case of death by heat or by  $\text{HgCl}_2$ , the rate of loss of reproduction was about twice as rapid as that of the loss of fermentation, about three times that of the loss of semipermeability, and about forty times

as large as the rate of coagulation. With ultraviolet light and with x-rays, these ratios were decidedly different.

The technique employed does not permit the conclusion that any one criterion of death is the prerequisite for other criteria. It does not appear probable that loss of reproduction is the prerequisite for loss of fermentation or of semipermeability because the ratios of the velocities of these processes are not the same with all causes of death.

There is no evidence that cells may show certain criteria of death immediately after exposure, and recover later.

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## ELECTROKINETIC PHENOMENA

### XI. ACTION OF UNI-UNIVALENT ELECTROLYTES ON ELECTRIC MOBILITY OF PROTEINS

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#### INTRODUCTION

The electric mobility of an ion in an aqueous salt solution decreases with increase in salt concentration, and in the same way, the electric mobility of particles of any size,—whether a protein, a particle of colloidal gold, an oil droplet, or a blood cell,—also decreases if sufficient salt is added. Particles like protein molecules which seem to ionize by means of some special mechanism can be treated from a rather simple point of view when compared with the treatment of surfaces acquiring an electric charge by adsorption processes.

In this communication a theory is presented which, although incomplete, satisfactorily accounts for the depressing effect of certain uni-univalent electrolytes on the electric mobility of proteins.

#### *Theoretical*

*Dependence of Electric Mobility in General upon  $\sigma$  and  $\kappa$ .*—In connection with the effect of salts on the electric mobility  $v_m$  of particles large when compared with ions, it should be recalled (see especially Mueller (1)) that if the dielectric constant of the medium  $D$ , the radius of the particle,  $r$ , and  $\eta$ , the viscosity of the medium, are considered constant,<sup>1</sup>  $v_m$  can vary with either the surface charge density,  $\sigma$ , or

\* This work was aided by a grant to this Department by the Chemical Foundation.

<sup>1</sup> The somewhat questionable procedure of using for values of  $D$  that of the pure solvent is supported by two important series of experiments: (1) The substitution of the dielectric constant of the pure solvent has been successful in the



with the reciprocal of the effective thickness of the double layer,  $\kappa$ , or with both. That is, in general, with the possibility that either  $\sigma$  or  $\kappa$  remain constant,

$$v_m = v_m(\sigma, \kappa) \quad (1)$$

*Inert Surfaces.*—Surfaces other than protein, like quartz, glass, cellulose, and paraffin oil, have electric mobilities which depend (in a rather complicated way) upon the concentration,  $c$ , of salts not reversing the sign of charge: the  $v_m$ - $c$  curves pass through a maximum (2), the height of which is frequently pronounced and is dependent upon the nature of the salt.<sup>2</sup> Abramson and Mueller (3) have shown

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case of experiments testing the Debye theory of electrolytes. (2) In the treatment of the problem by Mrs. Daniel use of the dielectric constant of the solvent (*J. Gen. Physiol.*, 1933, 16, 457) has predicted correctly changes in charge from protein mobility when  $D$  was lowered. See also Wyman, J., *J. Biol. Chem.*, 1931, 90, 443, and Weber, R., *Z. phys. Chem.*, 1931, 70, 711.

<sup>2</sup> That a maximum in the  $\zeta$ - $c$  curve is always observed where ion adsorption appears to be the mechanism of charge is not surprising in view of the relationships between  $\zeta$ ,  $\sigma$ , and  $c$ , as in equations (2), and (2a) (Stern; Gyemant). It is rather surprising, however, to find that quite large specific ionic effects are exhibited by salts like the alkali halides having a common negative ion,  $\zeta$  decreasing,  $\text{Li} > \text{Na} > \text{K} > \text{Rb} > \text{Cs}$  on cellulose and on glass; for salts with varying negative ions the order  $\text{I} > \text{Br} > \text{Cl}$  is observed. While it is true that the order in both cases is in the order of changing ionic electric mobility, the differences between the ionic mobilities are not sufficiently great to warrant the viewpoint that some mechanism connected primarily with the mobility of the ions is concerned. It seems more likely that in very dilute solutions where the maxima of the  $\zeta$ - $c$  curves are observed,  $\zeta$  could be built up by two processes of equal magnitude; (1) ionic adsorption, (2) polarization of the adherent molecules of water at the surface. Water molecules, consisting of dipoles as they do, should be attracted and oriented by the fields of the surrounding ions. As a quantitative measure of the effect of ions on the water dipoles, it is convenient to adopt the viewpoint that the heat of hydration of the gas ions is connected with the variation in  $\zeta$  at the maxima. Thus the ratio of the heats of hydration of the gas ions, ( $\text{Li}^+150$ ), ( $\text{Na}^+117$ ), ( $\text{K}^+97$ ), ( $\text{Rb}^+92$ ), ( $\text{Cs}^+86$ ), and ( $\text{Cl}^-63$ ), ( $\text{Br}^-52$ ), ( $\text{I}^-41$ ), give a plausible picture as follows. The positive ions should produce over a time average a greater increase in the orientation of negative charges and the surface so that  $\zeta$  should, other things being relatively equal, decrease

$$\text{Li} > \text{Na} > \text{K} > \text{Rb} > \text{Cs};$$

that the  $\sigma$ - $c$  curves (calculated from available data by means of the Debye theory) practically always follow a relatively simple course, for all salts not producing charge reversal; in each instance the curve resembled typical adsorption curves of the simple form described by Langmuir, with  $\sigma$  reaching a limiting value at about  $c = 0.01$  molar. Mooney (4) had previously published somewhat similar curves. The  $v_m$ - $c$  curve follows a complex course because both  $\sigma$  and  $\kappa$  are changing, the maximum resulting from changes in both as predicted by Stern.

*Proteins.*—Dissolved proteins and protein surfaces in different concentrations of uni-univalent salt solutions, like gelatin and egg albumin, represent a second group which differs in behavior in several important ways from the "inert" surfaces just discussed. The charge in the absence of salts seems to depend mainly upon the pH, for at any given pH a certain number of hydrogen ions over a time average are attached to the protein molecule. In the special case under discussion, of a uni-univalent salt not shifting the isoelectric point, we can first for simplicity consider  $v_m$  to depend only upon  $\kappa$ , if the pH is fixed. The significance of this result is evident when we consider the Debye-Henry (5) approximation, for the potential,  $\zeta$ , at the surface,

$$\zeta = \frac{Q}{Dr(\kappa r + 1)}, \quad (2)$$

where

$$\zeta = \frac{1}{6\pi} \frac{\eta v}{DX}, \quad (2a)$$

(for  $\kappa r > 30$  within experimental error),

and

$$Q = 4\pi r^2 \sigma.$$

and for the negative ions, the negative charges of the water dipoles now being repelled,  $I^-$  repelling less than  $Br^-$  and  $Cl^-$  so that for  $\zeta$  the order

$$I^- > Br^- > Cl^-$$

is obtained.

(Fajans, K., *Verhandl. deutsch. Phys. Ges.*, 1919, **21**, 549, 709, 714. Haber, F., *Verhandl. deutsch. phys. Ges.*, 1919, **21**, 750. Taylor, H. S., *Physical chemistry*, New York, D. van Nostrand Co., Inc., 2nd edition, 1931, 337.)

$\zeta$  and consequently  $v_m$ , which is proportional to  $\zeta$ , will depend only upon  $\kappa$  if all other terms are considered constant. Equation (1) now takes the form

$$v_m = v_m(\kappa)_{\sigma_{\text{pH}} = \text{constant}} \quad (3)$$

By assuming that  $\sigma$  remains constant we do not by any means imply that no change in  $\sigma$  occurs incidental to changes in  $\kappa$ . It is merely postulated that the change in  $v_m$  with  $\sigma$  due to  $\kappa$  varying is very small compared with the change in  $v_m$ , due to explicit variation of  $\kappa$ . Addition of salt, under these conditions, then, should, from equations (2) and (2a), cause only a diminution in  $v_m$  without a maximum in the curve.<sup>3</sup>

In Paper VI of this series (6) we have published an empirical equation in the form of equation (1),

$$\zeta = \frac{Q}{Dr(\kappa r + 2.4)}, \quad (4)$$

or

$$\zeta = \frac{Q}{(Dr) f(\kappa r)} \quad (4a)$$

Using equation (4a) we have plotted in Fig. 1, by evaluating  $f(\kappa r)$  for  $r = 4 \times 10^{-7}$  cm. and various values of  $v_m$  for gelatin (see Fig. 3), the theoretical form of the  $v_m$ - $c$  curves. Note the following points of interest in these curves:

1. The curves should in reality not cut the ordinate at  $c = 0$ , for in order to fix  $Q$ , a certain amount of acid must be present even though

<sup>3</sup> This approach differs somewhat from that of Sørensen, Linderstrøm-Lang, and Lund who have investigated the alteration of shape and direction of the ionization curves (not the electric mobilities) of ampholytes capable of reacting only with hydrogen ions. They find that the salt effect in accordance with their theory and in qualitative agreement with experiment, consists "in a turning of the ionization curves, indicating the relation between the quantity of combined acid (specific hydrogen ionization) and  $\text{pH}$ , and the turning of the curves which leaves the isoelectric reaction unaltered, tends in such a direction that the quantity of combined acid at constant  $\text{pH}$  increases with increasing salt concentration." On the other hand they find that the  $\text{pH}$  at which the quantity of acid (base) combined with egg albumin is 0, is independent of the salt concentration.

the concentration of protein and of salt is vanishingly small. In other words  $v_m$  is always accompanied by a finite value of  $\kappa$  which is given for strong acids by the concentration, not the mean activity of the

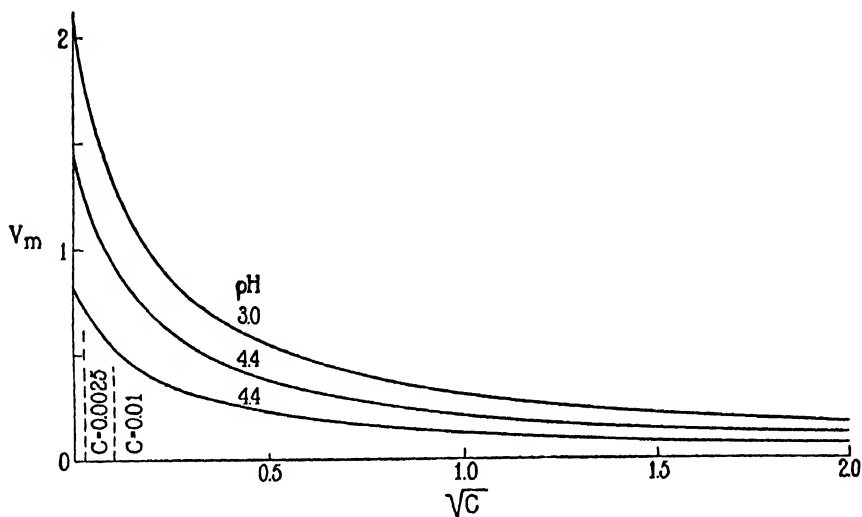


FIG. 1. Theoretical  $v_m$ - $c$  curves for gelatin at different values of pH, based upon single values given in Fig. 2. The two short vertical dotted lines at the lower left corner show the limits of extrapolation when the pH is sufficiently low to have appreciable amounts of acid present.

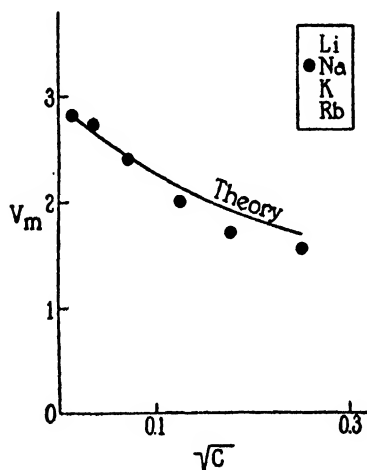


FIG. 2. Data of Loeb on particles of denatured egg albumin in 0.0002  $M$  NaOH. Loeb found no important difference in the effect of the alkali halides. The smooth curve is calculated by means of the theory here proposed, based upon the highest value of  $v_m$  found by Loeb.

acid. The dotted lines indicate, for example, the limiting position of the ordinate for  $c = 0.0025$  M, and  $c = 0.01$  M.

2. In more concentrated salt solutions the validity of equation (2) decreases; however, the curves indicate that  $v_m$  is probably still quite large even in 4 M salt solutions. Technical difficulties at present prevent measurements of  $v_m$  in salts of this concentration; but values of the proper magnitude have been observed by Hitchcock (7) in M/10 acetate buffers for gelatin and by the author for serum proteins (8) in solutions where  $c$  was equivalent to M/7. It would be most important to devise methods designed to discover if the available form of the theory is confirmed in that  $v_m > 0$  in concentrated salt solutions.

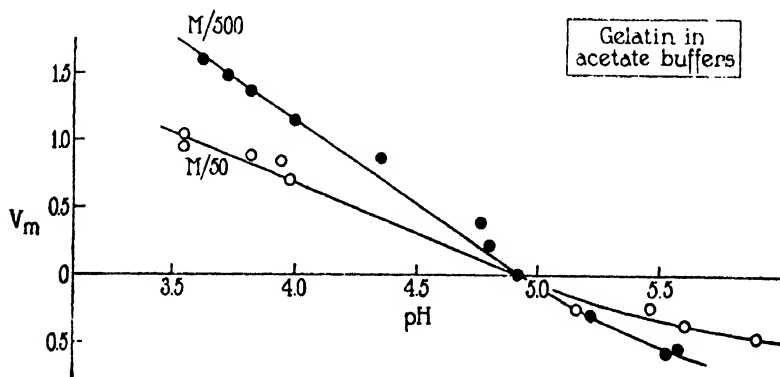


FIG. 3. The electric mobility of gelatin-coated quartz particles in acetate buffers. See text.

**Methods.**—As usual the Northrop-Kunitz (9) microelectrophoresis cell was employed for measurements of electric mobility. Values of pH are referred to  $\text{pH} = 1.07$  for N/10 HCl.

#### EXPERIMENTAL

(a) *Heat-Denatured Egg Albumin.*—In Fig. 2 the points are values of  $v_m$  given by Loeb (10) for denatured egg albumin particles in M/5000 NaOH. At this pH the electric mobility of the protein is rather high in the absence of salt and equal to about  $2.8\mu$  per second. The smooth curve is the theoretical curve for the influence of salts calculated by means of equation (4), taking  $r = 2.17 \times 10^{-7}$  cm. (11), and making the necessary assumption in regard to  $\eta$  and  $D$ . It is noteworthy that the course of the theoretical and experimental curves is almost iden-

tical, the actual difference being at the limits of the experimental error. Of interest is the fact, reported by Loeb, that within the limits of error (about 10 per cent) there was no difference in the action of the chlorides of Li, Na, Rb, and Cs. This is in accord with the simple theory, in contrast to the behavior of inert surfaces. The charging processes of the latter seem to be unrelated to phenomena easily associated with stoichiometrical reactions. The charging process in the simplest protein case considered in the foregoing should be determined only by the hydrogen ion activity. This will probably be found to be not true, in general, for some process tantamount to adsorption of ions of the salt may occur, the shape of the  $v_m$ - $c$  remaining essentially the same but shifted because of the effect of salts on the isoelectric point.

(b) *Gelatin*.—Since, as Hitchcock (7) has shown, sodium acetate buffers of different concentrations do not change the isoelectric point of inert particles covered with gelatin, it was considered desirable to report the effect of acetate buffers of different concentrations on the electric mobility of quartz particles with an adsorbed gelatin film. Coignet gelatin was used in order to compare our results with those of Prideaux and Howitt (12) on gelatin-covered particles. In Fig. 3 are given our electric mobility data in  $M/500$  and  $M/50$  sodium acetate buffers.<sup>4</sup> Note that the shapes of the  $v_m$ -pH curves are essentially unchanged on increasing  $c$ . To demonstrate this, Curve II has been plotted as  $5/8$  of each ordinate value of Curve I. The mobility curve is, therefore, simply rotated about the isoelectric point by increasing the salt concentration. The value of  $\kappa$  changes from  $\kappa = 0.15 \times 10^7$  in  $M/500$  salt to  $\kappa = 0.47 \times 10^7$  in  $M/50$  salt. For reasons given in Paper VI,  $r$  is to be taken not as that of the quartz particles, but as that of the dissolved molecules. To obtain a value of an equivalent radius our empirical form of equation (1) has been solved for  $r$ ,  $Q$  being known from the titration curve of the gelatin (13).

To obtain some idea of the equivalent radius of gelatin it is necessary to proceed by a somewhat unsatisfactory course because gelatin solu-

<sup>4</sup> Prideaux and Howitt investigated  $v_m$  for colloidal gold particles covered with an adsorbed gelatin film. Using the moving boundary method, they did not find the effect of salts as marked as that reported here. However, all of our data on quartz particles have agreed with other data obtained with the moving boundary method by Tiselius and by Koenig and Pauli.

tions are polydisperse. At pH = 4.0 in acetate buffers the average molecular weight of gelatin is about 35,000 with little change on aging. The adoption of this value will enable us to obtain a somewhat dubious value of an equivalent radius (assuming a spherical molecule) from equations (3a) and (4),

$$Q = 6 \pi \eta r v_m (\kappa r + 2.4).$$

The substitution of values for  $Q$ ,  $v_m$ , and  $\eta$  in this equation (3) yields  $r = 4 \times 10^{-7}$  cm.

We are now in a position to test on gelatin surfaces our assumption that the addition of salt produces a diminution in  $v_m$  because of a change in the double layer rather than a change in charge. The data in Fig. 3 are for changes in  $\kappa$  as previously mentioned. Then if the increase in salt does not change  $Q$  appreciably, we have, from equations (2) and (3),

$$\frac{v_m}{v'_m} = \frac{(\kappa' r + 2.4)}{(\kappa r + 2.4)},$$

or substituting, we have predicted the ratio for the data in Fig. 3,

$$\frac{(1.88 + 2.4)}{(0.6 + 2.4)} = 1.4, \text{ approximately.}$$

This is in agreement with that ratio found, 1.6, at the limits of error.

Note that the choice of 35,000 as the molecular weight does not sensibly change the general result. If 17,500 had been chosen the ratio of the mobilities would have come out close to 1.3; if 70,000 had been chosen the ratio would have been closer to 1.6.

This agreement, in the case of unsatisfactory material like gelatin, further justifies the assumption that the salt decreases the potential of this protein, accounting for the change in  $v_m$  which is proportional to the change in potential.

(c) *Egg Albumin*.—The data of Tiselius and of Abramson for egg albumin given in Paper VI of this series are given in Curve I of Fig. 4a. These experiments were performed in M/50 sodium acetate buffers ( $\kappa$  being held constant). In order to test the usefulness of our empirical equation, the dotted curve (theoretical) was plotted for M/1000 sodium acetate buffers ( $r = 2.17 \times 10^{-7}$  cm.) assuming no

shift in the isoelectric point. Experimentally, however, it was found that under our conditions the isoelectric point of the egg albumin in

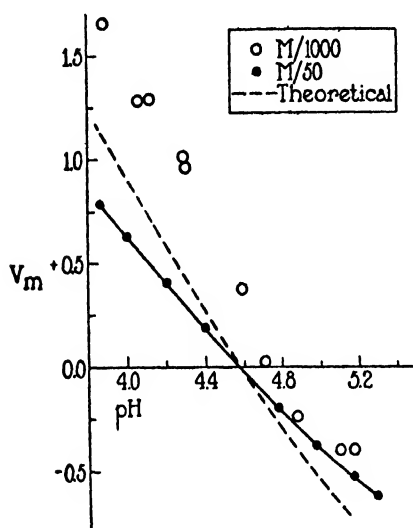


FIG. 4a

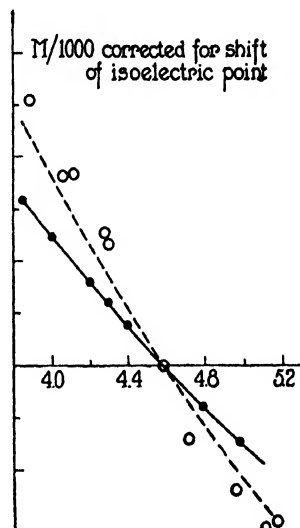


FIG. 4b

FIG. 4a. The effect of the concentration of acetate buffers on the magnitude of  $v_m$  (egg albumin) and on the position of the isoelectric point.

FIG. 4b. By correcting for the shift in the isoelectric point, the effect of diluting the buffer on values of  $v_m$  is predicted.

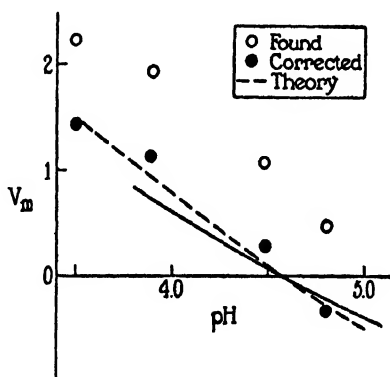


FIG. 5. The electric mobility of egg albumin in HCl solutions treated as in Figs. 4a and 4b.

acetate buffers was shifted about 0.2 pH to  $\text{pH} = 4.8$ . These data are plotted as open circles, the data running parallel to the theoretical



curve. This complicates the problem somewhat, for it is necessary to introduce some correction for the shift in the isoelectric point due to the change in total salt. Assume that the total number of anions and cations as well as their ratio and their charge may influence the dissociation of the protein. On decreasing the salt concentration as we have done in the acetate buffer experiments on gelatin, the isoelectric point was not shifted. The isoelectric point is shifted in the analogous experiment with egg albumin. If we wish to visualize this effect by assuming that the dissociation of the protein depends upon the total salt, and that this effect is independent of the pH, it is reasonable to correct this shift in order to have comparable values of  $Q$  due to hydrogen ions by shifting the  $v_m$ -pH curve in  $M/1000$  acetate solutions as a whole, so that the isoelectric points coincide. This has been done and the result depicted in Fig. 4*b*. The open circles are the actual data corrected as just proposed. This correction is merely the addition, to each value of  $v_m$  in  $M/1000$  acetate, of  $-0.37\mu$  per second, the difference in the two buffers at  $pH = 4.6$ . This correction results, as demonstrated in the Fig. 4*b* in the open circles falling on the theoretical curve within the limits of experimental error. Fig. 5 depicts the data for adsorbed egg albumin in HCl. The values of  $v_m$  have been treated as in the foregoing, with the theory confirmed.

#### SUMMARY

By assuming that the electric charge of proteins is primarily determined by the hydrogen ion activity of the medium, and by making corrections when necessary for the effect of salt, it is possible to derive a simple relationship between the electric mobility of proteins and the effective reciprocal thickness of the electric double layer. The decrease in electric mobility of proteins in solutions is readily predicted for gelatin in acetate buffers and for egg albumin in the presence of the alkali halides, of acetate buffers, and of hydrochloric acid on the basis of the assumptions made.

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## OSMOTIC RELATIONSHIPS IN THE HEN'S EGG

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Since Straub's observation that an osmotic pressure difference of two atmospheres was maintained between the yolk and white of the hen's egg by the apparent application of metabolic energy, considerable doubt has been cast upon the actual existence of such a difference by those who have found that the osmotic pressure of the yolk and the white is the same. Although it has been admitted that there has been a considerable amount of striking evidence in support of Straub's findings, the claim has been made that while the experimental technique employed in obtaining these data might be applied to dilute aqueous solutions, it could not be applied to a material such as egg yolk for the purpose of obtaining accurate measurements.

The present paper contains data which were obtained in a critical study of the technique involved in determining the osmotic pressure of egg yolk, of egg white, and of a mixture of these, by the freezing point method. These data could be obtained consistently and are therefore believed to be accurate. Without exception they are in support of Straub's observation that there is a considerable difference in osmotic pressure between the yolk and the white of newly laid eggs. The difference in osmotic pressure between the yolk and the white of newly laid eggs has led to the interesting postulation of a vital activity at the yolk membrane which was believed to maintain this osmotic difference by doing work. Evidence obtained in the present investigation is however in support of the point of view of Smith and Shepherd who found that osmotic equilibrium between the yolk and white is slowly established and that the postulation of a vital activity maintaining a steady state is therefore unnecessary.

A review of similar work which has been done in the past is contained in a recent paper by Howard (2), who reports results obtained

in determining the osmotic relationship in the hen's egg, partly with the use of the freezing point method devised by the writer (3). From these data Howard concludes that "the postulate of a vital activity at the yolk membrane maintaining an osmotic pressure difference is thus shown to be unnecessary, since a simple osmotic equilibrium exists between the yolk and the white," and that certain previous observations of other investigators who found a difference in osmotic pressure, "were in error due to their failure to recognize that the determination of the true freezing point of egg yolk is beset with technical difficulties."

At the time when the writer devised the freezing point method used by Howard, he employed it in measuring the freezing point of egg yolk and of egg white. The expectation of finding that previously reported freezing point differences between these might be due to experimental errors was however not realized for it was found in the limited number of preliminary determinations<sup>1</sup> which were made that a difference exists. Howard's more recent results with this method, showing that a difference is lacking, have however led the writer to carry out a more complete series of determinations.

### *Method*

During the course of a series of more than one hundred freezing point determinations on egg yolk, on egg white, and on a mixture of these, the following technique was found to give consistent and apparently reliable results.

After the yolk had been separated from the egg white it was moved about on a  $\frac{1}{4}$  inch mesh screen grid which had been coated with hard paraffin wax. Considerable amounts of egg white which had remained with the yolk were thus removed by forming a film between the meshes of the screen. Small gelatinous lumps adhering to the yolk were removed with the tip of a pipette to which suction was applied. The yolk was then stirred with a glass rod and after the vitelline membrane had been isolated from it, it was used immediately for a freezing point determination.

The main bulk of the egg white was stirred with a motor-driven stirrer made by attaching a rubber-tipped glass rod to a high speed motor. A smaller amount (10 to 12 ml.) was then placed into a narrower tube (16 to 17 mm. in diameter) and stirred 2 minutes longer. The formation of froth was avoided as much as possible. The fluid thus obtained could be poured drop by drop. It was kept packed in melting ice and used almost at once for a freezing point determination.

When a mixture of white and yolk was required, a 12 ml. portion of approxi-

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<sup>1</sup> Unpublished results.

mately equal parts of egg white and yolk was further stirred at high speed for 2 to 3 minutes in the narrower tube before being used for a freezing point determination.

The eggs were used on the same day on which they were laid.

The apparatus used in making the freezing point determinations was the same as that previously described (3) except that the innermost tube, *F*, containing the charge and the thermometer, was 16 mm. in diameter. Since sufficient amounts of material were available, 8 ml. of fluid were used, instead of 1 ml., in a single determination.

The temperature of the freezing bath was maintained at  $-1.0^{\circ}$ , and controlled by occasionally dropping in small pieces of dry ice. The part of the apparatus consisting of tubes *E* and *F*, containing the experimental material and the thermometer, was cooled to about  $+0.5^{\circ}$  in melting ice before it was placed into the tube *D* of the apparatus. It was then further cooled by periodically sucking the liquid from the tube *D* into the tube *E* until the temperature of the experimental fluid was about  $0.1^{\circ}$  above that of the anticipated freezing point. Further cooling was then allowed to take place without bringing the liquid in *D* in contact with the tube containing the experimental fluid. The experimental fluid was stirred by moving the thermometer *H*, up and down, at the same time giving it a rotating motion along the wall of its container.

When the egg yolk had been supercooled  $0.10$ – $0.15^{\circ}$  it was seeded six to eight times in the course of 2 minutes and well stirred after each seeding. Stirring was continued intermittently for 6 minutes from the time of the initial seeding. Temperature readings were then taken at 1 minute intervals for a period of 10 minutes, the thermometer being tapped just before each reading. If a constant temperature plateau was not reached within 4 to 5 minutes after readings were begun (*i.e.* within 10 to 11 minutes after the initial seeding) the inner tube was again placed in melting ice, until all of the ice in the experimental fluid had melted, and the freezing point determination commenced anew. It was found, when the experimental fluid was not stirred too vigorously and was properly seeded, that readings at equilibrium did not decrease more than  $0.0005^{\circ}$  per minute. The earliest readings of the constant temperature plateau were accepted as the true freezing point values.

In the case of egg white or of a mixture of egg white and yolk the fluid was similarly seeded, as above, when it had been supercooled  $0.05$ – $0.10^{\circ}$  below the anticipated freezing point, but stirring was discontinued 3 minutes after the initial seeding. Readings were begun when stirring was discontinued and were taken every minute for a period of 10 minutes. Readings generally dropped to a constant temperature plateau within 3 or 4 minutes. A constant temperature plateau was less readily obtained with egg white than with yolk or with a mixture of egg white and yolk. When a constant temperature plateau was not obtained the material was warmed in melting ice and the freezing point determination begun anew. Readings were made with the use of a reading glass.

*Discussion of Technique*

The nature of egg yolk makes prolonged stirring for the purpose of rendering it homogeneous unnecessary. Egg white is, however, so stringy and lumpy that proper seeding and stirring within the apparatus could not take place unless it had been well disintegrated previously. In the case of a mixture of egg white and yolk thorough mixing is also essential as otherwise the white will freeze before a state of osmotic equilibrium between yolk and white has been attained.

TABLE I

Egg yolk		Egg white	
Time	Temperature	Time	Temperature
<i>min.</i>	°C.	<i>min.</i>	°C.
0	Seeded	0	Seeded
6	-0.540	3	-0.453
7	-0.565	4	-0.452
8	-0.570	5	-0.452
9	-0.573	6	-0.452
10	-0.575	7	-0.452
11	-0.575	8	-0.452
12	-0.575	9	-0.452
13	-0.576	10	-0.453
14	-0.576	11	-0.453
15	-0.576	12	-0.453
16	-0.576	13	-0.453
		14	-0.453
		15	-0.453

The failure to properly mix the egg white and yolk was found in the present investigation to give freezing points for the mixture which were near those of the white. A similar circumstance might well be held accountable for results obtained by others in the past when it was found that the freezing point of the mixture was approximately that of the white. Thorough mixing, however, was found to give freezing points for the mixture which were quite distinct from those of either yolk or white. The following is an illustration of this relationship.

Freezing point of egg white  $-0.427^{\circ}$ , of yolk  $-0.545^{\circ}$ , of a mixture of equal parts  $-0.461^{\circ}$ .

The data given in detail in Table I are representative of a successful determination. Although such results were not invariably obtained on a first trial, acceptable results could be readily obtained, so that, after a considerable experience with the technique, the greater percentage of determinations had to be made but once.

Aside from temperature control, proper stirring and uniform seeding are perhaps the most important parts of the technique. Egg yolk is so viscous that superfluous stirring will result in heating local areas

TABLE II

(1)		(2)	
Time	Temperature	After warming in melting ice	
		Time	Temperature
<i>min.</i>	$^{\circ}\text{C.}$	<i>min.</i>	$^{\circ}\text{C.}$
0	Seeded	0	Seeded
6	-0.495	6	-0.525
7	-0.540	7	-0.545
8	-0.550	8	-0.550
9	-0.558	9	-0.554
10	-0.560	10	-0.555
11	-0.561	11	-0.555
(Stirred 1 min.)		12	-0.556
12	-0.450	13	-0.556
13	-0.525	14	-0.557
14	-0.545	15	-0.557
15	-0.553		
16	-0.559		
17	-0.563		
(Results were rejected and a new determination made)			

above the freezing point. The yolk nearest the thermometer would thus contain no ice crystals while other areas would still be below the freezing point and would contain ice crystals. The distribution of ice crystals throughout the whole mass requires a considerable amount of moderate mixing. Egg white, even after vigorous stirring, may on the other hand still be relatively lumpy in comparison with the average aqueous solution. A reasonable amount of stirring at high speed before the material is used as well as thorough mixing in the



freezing point apparatus is therefore necessary to prevent the localization of seeded areas. The danger of overheating by stirring need not be taken into account in the case of egg white as in the case of the yolk. The lack of uniform seeding, due to superfluous stirring (in case of the yolk, to insufficient stirring, or to a lumpy material (in case of the white) appears to permit the localization of areas which are super-

TABLE III  
*Data Obtained with Egg Yolk*

Time	Temperature	Temperature (after remelting in ice)	Temperature (after remelting in ice)
<i>min.</i>	$^{\circ}\text{C.}$	$^{\circ}\text{C.}$	$^{\circ}\text{C.}$
0	Seeded	Seeded	Seeded
6	-0.455	-0.530	-0.535
7	-0.505	-0.537	-0.539
8	-0.533	-0.540	-0.542
9	-0.549	-0.540	-0.544
10	-0.559	-0.541	-0.545
11	-0.568	-0.541	-0.545
12	-0.572	-0.542	-0.546
13	-0.574	-0.542	-0.546
14	-0.572	-0.542	-0.547
15	-0.570	-0.543	-0.547
16	-0.571	-0.543	-0.548
17	-0.569	-0.543	-0.548
18	-0.568		
19	-0.567		
20	-0.569		
21	-0.571		
22	-0.574		
23	-0.575		
24	-0.575		
25	-0.575		

cooled. Data which are on a constant temperature plateau cannot be obtained under such conditions.

An illustration of such an occurrence is shown in Table II. The data were obtained with egg yolk.

The results in the first column of Table III show that a delayed constant temperature plateau, obtained after a prolonged period of temperature fluctuations, represents freezing point values which are not

acceptable. The point of equilibrium is much lower than that of the second column. The data of the second column could be reproduced within a few thousandths of a degree as shown by the data of the third column. Data such as those of the last two columns are not as uniform as can be obtained occasionally, but are considered acceptable since they can be readily obtained and do not vary more than  $0.0005^{\circ}$  per minute once a temperature plateau has been reached.

### *Freezing Point Results*

The data of Table IV were obtained with eggs from two separate flocks of white Leghorns. Both were extraordinarily large flocks

TABLE IV

	Freezing point of yolk	Freezing point of white	Difference
Fertilized eggs			
	$^{\circ}\text{C.}$	$^{\circ}\text{C.}$	$^{\circ}\text{C.}$
No. 1	-0.550	-0.425	-0.125
" 2	-0.545	-0.427	-0.118
" 3	-0.545	-0.410	-0.135
" 4	-0.539	-0.415	-0.124
Unfertilized eggs			
No. 1	-0.555	-0.455	-0.100
" 2	-0.555	-0.450	-0.105
" 3	-0.560	-0.446	-0.114
" 4	-0.556	-0.430	-0.126
" 5	-0.575	-0.452	-0.123

maintained for the wholesale production of eggs. One flock produced fertile eggs and the other unfertile eggs.

### DISCUSSION

The data of Table IV show a constantly pronounced difference between the freezing points of the yolk and the white of the newly laid hen's egg. That this osmotic difference is not maintained but gradually equalizes itself was shown by the results of Smith and Shepherd (4), who not only postulated but found a slow diffusion to take place between the yolk and the white. The slow attainment of

a state of osmotic equilibrium between yolk and white, even when these have been mixed, was demonstrated in the present experiments. It was found that a mixture which had been moderately stirred had a freezing point not greatly different from that of the white alone, a fact which would indicate a lack of osmotic equilibrium between the two constituents. After prolonged stirring with a high speed stirrer such a mixture was found to have a freezing point which represented the average which a mixture of yolk and white at equilibrium might be expected to have. While this circumstance does not disprove the existence of a vital force at the vitelline membrane, it demonstrates the difficulty of attaining a state of osmotic equilibrium between yolk and white and at least explains the difficulty which some have had in obtaining a freezing point for the mixture of yolk and white which was different from that of the white alone. As has also been shown by the results obtained by Smith and Shepherd there appears to be no reason why complete osmotic equilibrium between yolk and white should not be attained after a sufficient length of time. The state which exists between the yolk and the white of the newly laid egg therefore does not require the postulation of a vital force for the maintenance of a difference in osmotic pressure, since the evidence is in favor of a slow equalization of the difference in osmotic pressure and a steady state does not appear to exist.

#### SUMMARY

Data have been given to illustrate the difficulty of obtaining consistent freezing point data with a viscous fluid such as the yolk of the hen's egg and a technique has been described for obtaining reproducible and accurate results consistently. Further freezing point data have been given which were obtained with both fertile and unfertile hen's eggs by the use of a freezing point method previously described by the writer. These data show that there is a pronounced difference between the freezing points of the yolk and the white in contrast to data obtained by the use of the same method by Howard who found the freezing points of the yolk and the white to be the same.

It was shown by freezing point determinations that even in a mixture of yolk and white osmotic equilibrium is slowly arrived at. This again emphasizes the fact established by Smith and Shepherd that

since osmotic equilibrium between yolk and white is slowly arrived at, the postulation of a vital activity at the yolk membrane is unnecessary, since the steady state previously postulated need not be assumed to exist.

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## CRYSTALLINE PEPSIN

### V. ISOLATION OF CRYSTALLINE PEPSIN FROM BOVINE GASTRIC JUICE

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A crystalline protein having powerful proteolytic activity has been described in previous papers of this series (1). This protein was isolated from commercial pepsin preparations which, in turn, had been prepared from the gastric mucosa of swine.<sup>1</sup> The possibility exists that this protein was formed from some more complicated compound during the process of extraction and does not represent the enzyme as secreted normally in the gastric juice. In order to determine whether the activity of the gastric juice is due to the same protein, experiments were undertaken to isolate the enzyme from gastric juice.

Preliminary determinations of the activity of swine gastric juice showed that if the activity were due to the crystalline enzyme isolated from the gastric mucosa, the quantity of this protein in the gastric juice was extremely small and that a very large quantity of gastric juice would be necessary before any attempt at isolation could be made. It was found to be impossible to obtain sufficient quantities of swine gastric juice but bovine gastric juice may be collected in quantity as described by Williams<sup>2</sup> (2).

The contents of the fourth pouch of cattle was removed as soon as possible after the cattle had been killed in the slaughter house. About  $\frac{1}{2}$  liter of juice was obtained from each animal and larger quantities were found in those animals which had been recently fed. The juice obtained in this way contained more or less undigested food and fil-

<sup>1</sup> The writer is indebted to Dr. F. O. Taylor of Parke, Davis and Company for this information.

<sup>2</sup> The writer's attention was directed to this valuable method by Dr. Robert Loeb of the Presbyterian Hospital.

tered with difficulty. After filtration it was found to contain about 1 mg. of nitrogen and about 0.002 proteolytic units per ml. as determined by the hemoglobin method (3). This corresponds to about 0.01 mg. of nitrogen in the form of the enzyme protein previously isolated, assuming that the activity of the enzyme in the gastric juice is the same as that of the purified enzyme (4). This quantity of protein is too small to be determined and in fact the gastric juice gives no precipitate with trichloroacetic acid and would ordinarily be considered to be protein-free.

Pekelharing (5) has described a method of obtaining a highly active protein from gastric juice by dialysis against dilute hydrochloric acid and this method was tried. It was found, however, that a large loss in activity occurred during the dialysis so that the yield of active material was too small to be practical for purposes of isolation. It is also evident from the value for the solubility of crystalline pepsin already determined (6) that it cannot precipitate from gastric juice under the conditions described by Pekelharing since the solubility of the crystalline pepsin was found to be about 0.3 mg. of nitrogen per ml. at the minimum point (pH 2.8) while from the value for the activity the gastric juice contains only about 0.01 mg. pepsin nitrogen per ml. There is no doubt, however, that an active precipitate appears upon dialysis, as described by Pekelharing, but it is probable that this precipitate consists of a compound or an adsorption complex of the enzyme, possibly with the mucin or a mucoprotein present in the gastric juice.<sup>3</sup> This conclusion is further indicated by the fact that the analysis of Pekelharing's preparation showed higher carbon and hydrogen and lower nitrogen than does crystalline pepsin itself.

### *Method of Isolation*

Fractionation with various salt solutions was tried and it was found that the activity could be concentrated in the protein fraction. This

<sup>3</sup> This mucilaginous material is probably the mucoprotein recently described by Webster and Komarov (*J. Biol. Chem.*, 1932, **96**, 133) who have suggested the possibility of the existence of a complex between this mucoprotein and the pepsin. This compound does not exist in solution, however, since the diffusion coefficient of the enzyme in the gastric juice is the same as that of the purified enzyme. These experiments are described later in the paper.

TABLE I

*Preparation of Crystalline Pepsin from Bovine Gastric Juice*

	No.	Vol.	N/ml.	[P.U.] <sup>Hb</sup>		
				Per ml.	Total	Per mg. N
		ml.	mg.			
Contents of 4th pouch removed immediately after death and filtered through fluted paper (S. and S. No. 1450 1/2) 48 hrs. 6°C. ....	1	5500	1.0	0.0025	14	0.0025
Filtrate saturated with ammonium sulfate, decanted and filtered with suction, precipitate dissolved in 200 ml. N/500 hydrochloric acid...	2	200				
Sol. No. 2 cooled to -10°C. and 300 ml. cold acetone added, centrifuged, supernatant. ....	3	500		0.018	9	
Sol. No. 3 cooled to -10°C. and 500 ml. cold acetone added, filtered, and precipitate dissolved in 300 ml. N/500 hydrochloric acid. ....	4	310		0.025	7.8	
Sol. No. 4 + 1 volume saturated magnesium sulfate, centrifuged, precipitate dissolved + 100 ml. N/500 hydrochloric acid. ....	5					
Sol. No. 5 + 1 volume saturated magnesium sulfate, centrifuged, precipitate dissolved + 100 ml. N/500 hydrochloric acid. ....	6	114	0.53	0.065	7.4	0.12
Sol. No. 6 + 1 volume saturated magnesium sulfate, centrifuged, precipitate dissolved + 70 ml. N/500 hydrochloric acid. ....	7					
Sol. No. 7 + 1 volume saturated magnesium sulfate, centrifuged, precipitate dissolved + 75 ml. N/500 hydrochloric acid. ....	8	80	0.42	0.078	6.3	0.186
240 ml. of solution No. 8, equivalent to 15 liters gastric juice + 1 volume saturated magnesium sulfate, filtered, precipitate. ....	9	3 gm. filter cake			20	
Precipitate No. 9 + 10 ml. N/10 sodium acetate, titrated to pH 3.0 + N/2 sulfuric acid, slight precipitate in dark viscous liquid, could not be filtered nor centrifuged; 1 volume saturated magnesium sulfate added and suspension filtered with suction. Precipitate dissolved in 8 ml. N/10 sodium acetate, clear yellow solution..	10	12	5.6	1.0	10	0.18
Titrated to pH 3.0 + N/2 sulfuric acid, stood 18 hrs. 6°C., filtered with suction (slow). Precipitate dissolved at 45°C. with minimum quantity of water. ....	11	10	2.8	0.5	5	0.18
Cooled slowly while stirring. Crystals formed after about 1 hr. Kept at 20°C. for 24 hrs. and filtered. Precipitate normal pepsin crystals slightly yellowish—about 0.1 gm. ....	12					
Crystals dissolved in N/10 sodium acetate. ....	13	20	0.77	0.146	2.8	0.19



fraction, however, contained a large amount of a mucilaginous substance which rendered the solutions extremely difficult to work with since they could be centrifuged or filtered only with the greatest difficulty. This difficulty has been encountered by other workers and it was found by Fenger and Andrew (7) that the active material could be freed from this mucilaginous impurity by precipitating in the cold with 75 per cent acetone. This process entails a loss of nearly half the total activity but no more satisfactory method could be found. A method of fractionation was eventually worked out, which consisted essentially in preliminary precipitation with saturated ammonium sulfate, solution in 60 per cent acetone, and precipitation with 75 per cent acetone. This process yielded a white amorphous precipitate free from most of the mucilaginous impurity and possessing about half of the total original activity. It was further purified by repeated precipitation with one-half saturated magnesium sulfate and finally crystallized from warm water. The details of the method are shown in Table I.

As the table shows, the activity per mg. of nitrogen, as determined by the hemoglobin method, increases from 0.0025, which is the value found in the original gastric juice, to about 0.19, which is the characteristic value already found for the crystalline protein isolated from swine (3). The yield of crystalline material is very poor and represents only a few per cent of the total original activity. Of this loss, about one-half occurs during the precipitation with acetone and the remainder during the repeated precipitation with magnesium sulfate. Actually about 100 mg. of crystalline material was obtained from 15 liters of gastric juice.

### *Properties of the Crystalline Protein*

#### *1. Crystalline Form*

The crystals are small, hexagonal, bipyramids and are indistinguishable by inspection from the crystals obtained previously from the gastric mucosa of swine.

#### *2. Specific Activity of the Crystals*

The activity of the material was determined, as previously described (4), by a series of methods and compared with that of a purified prepa-

ration from swine mucosa. No significant difference in activity could be detected by any of the methods. The results of these experiments are shown in Table II.

It will be noted that the specific activity of both preparations differs in some cases from that already described for the swine pepsin but that the activity of the two preparations, as determined in this series of experiments, was not significantly different. This disparity between the activity found in these determinations and that found previously is due to the fact that the protein solutions were different from those in the previous experiments and, as usual, gave slightly different figures for the activity. The activity, as determined by the digestion of

TABLE II

*Properties of Crystalline Pepsin from Bovine Gastric Juice and from Swine Stomach*

Pepsin	Proteolytic activity per mg. nitrogen										
	Gel. V <sup>-</sup>	Cas. V <sup>-</sup>	Ed. V <sup>-</sup>	Cas. V <sup>+</sup>	Rennet	Cas. S	Ed. S	Cas. F	Ed. F	Gel. F	Hb
Bovine gastric juice	10.6	1300	640	430	170,000	0.27	0.20	0.16	0.072	0.0008	0.180
	10.8	1400	620	450	165,000	0.26	0.22	0.09	0.075	0.0007	0.182
Swine stomach standard pepsin solution May 9, 1932	11.0	1400	625	500	168,000	0.30	0.22	0.11	0.075	0.0007	0.183

hemoglobin, however, agrees with that previously found since hemoglobin solutions are more reproducible than those of the other proteins.

Since only a small per cent of the original activity present in the gastric juice was actually isolated in the form of the crystalline protein it is possible that the original gastric juice contains other enzymes and that only a part of its activity is due to the fraction isolated. In order to check this point the activity of the gastric juice, as determined by various methods, was compared with the activity of crystalline swine pepsin. Since the gastric juice is too dilute for protein nitrogen determinations to be made, the specific activity per mg. of protein nitrogen cannot be calculated and it is necessary to express the results in terms of the activity per ml. solution. If the gastric juice contained other proteolytic enzymes it would be expected that the relative activity of the juice, compared to swine pepsin, would vary when

measured with different proteins. This, however, is not the case. If a solution of crystalline pepsin is made up so as to have the same activity per ml. as the gastric juice, as determined by the hemoglobin method, the two solutions are found to have the same activity when the activity is determined by any of the other methods. If the gastric juice, therefore, contains another enzyme beside that isolated, the relative activity of this hypothetical enzyme on the various proteins must be the same as that of the fraction isolated.

### *Optical Activity*

The optical rotation of the material was determined in solution in  $N/10$  sodium acetate, pH about 5.2 at 25°C. with the  $D$  line. The specific rotation was found to be about  $-93^\circ$  while recrystallized swine pepsin gave a specific rotation of about  $-70^\circ$ . However, the optical activity of swine pepsin, which has only been crystallized once, varies from  $-100^\circ$  to  $-80^\circ$  so that the difference cannot be considered significant. Lack of material prevented further purification of the bovine pepsin. This variation in the optical activity is probably due to the presence of non-protein impurities.

### *Diffusion Coefficient*

It was mentioned in discussing the method of isolation that a mucilaginous substance from which the active protein can be separated only with great difficulty is present in quite large amount in the original gastric juice. The possibility is suggested, therefore, that the enzyme is present in the gastric juice in some form of combination with this mucilaginous material. The methods used in the isolation would not be expected to split any chemical compound but might be considered to separate an adsorption complex. If such an adsorption complex existed in the gastric juice it would be expected that the size of the active particles (or molecules) in the juice would be considerably larger than that of the molecules of the purified enzyme. The diffusion coefficient of the active substance in the gastric juice should therefore be smaller than that of the purified enzyme. In order to determine the diffusion coefficient and, hence, the size of the active molecule in the unchanged gastric juice, the measurements were carried out as already described for the swine pepsin (8, 9) but owing

to the low concentration of enzyme present in the gastric juice they could not be made as accurately. The measurements gave a value for the diffusion coefficient in  $N/1$  pH 4.5 acetate buffer at  $6^{\circ}\text{C}$ . of  $0.049 \pm 0.002 \text{ cm.}^2/\text{day}$  which agrees, within the experimental error, with that previously found (9) for the crystalline swine pepsin. These measurements are experimental evidence that the active molecule present in the gastric juice is the same or very nearly the same size as that in a solution of the purified crystalline protein and hence that the active molecule in the gastric juice does not exist in the form of a large complex.

### *Solubility Experiments*

The experiments just described show no significant difference in properties between the bovine pepsin and the swine pepsin. A much more sensitive test for the identity of the two, however, consists in solubility measurements since the solubility of even closely related proteins is quite characteristic. This method, theoretically, is the same as the classical melting point method of organic chemistry and was first used as a method of establishing the difference between similar proteins by Landsteiner and Heidelberger (10). The solubility of two different substances is, in general, independent of each other so that the solubility of a mixture of two different solids would be equal to the sum of the two solubilities separately. If the two solids, however, form a solid solution the solubility of a mixture of the two cannot be predicted with certainty but would be expected to lie between that of the two substances in pure solution (11). This result was obtained by Landsteiner and Heidelberger with hemoglobins from closely related animals and indicates that the hemoglobins are different but form solid solutions rather than mixtures. Owing to the small amount of material available it was not found possible to obtain accurate solubility figures for the bovine pepsin since it was necessary to use a precipitate obtained from the mother liquor of the crystallization. This mother liquor was precipitated with half-saturated magnesium sulfate, the precipitate redissolved in 0.02 molar pH 4.65 acetate buffer and precipitated by the addition of an equal volume of magnesium sulfate solution having a specific gravity of 1.294. This precipitate was stirred with about 10 ml. of a solution consisting of 1 volume of 0.02 molar pH 4.65 acetate and 1 volume saturated magnesium sulfate

specific gravity 1.294, the suspension centrifuged, and the supernatant solution analyzed for nitrogen. This process was repeated until a constant value was obtained on successive washings. Four or five washings are usually required before constant solubility is obtained. The precipitate was then dissolved by the addition of 5 ml. of 0.02 molar pH 4.65 acetate and precipitated by the addition of 5 ml.

TABLE III

*Solubility of Pepsin from Swine Mucosa and from Bovine Gastric Juice*

in  $\left\{ \begin{array}{l} 0.01M \text{ pH } 4.65 \text{ Acetate} \\ 0.50 \text{ Saturated Magnesium Sulfate} \end{array} \right\}$  at  $26^{\circ}C$ .

Specific gravity saturated magnesium sulfate  $\frac{22^{\circ}}{H_2O \ 22^{\circ}} = 1.294$

	No.	Swine pepsin		Bovine pepsin		Swine + bovine	
		n/ml.	Hb [P. U.] <sub>ml.</sub>	n/ml.	Hb [P. U.] <sub>ml.</sub>	n/ml.	Hb [P. U.] <sub>ml.</sub>
		mg.		mg.		mg.	
About 1 gm. amorphous pepsin stirred with 10 ml. solvent, centrifuge, and filter, repeat 4 times, filtrate. ....	1	0.80	0.17	0.69	0.12		
5 ml. 0.02 pH 4.65 acetate added to precipitate, clear solution. ....							
5 ml. sat. magnesium sulfate added (precipitate), centrifuge and filter supernatant. ....	2	0.84					
5 ml. supernatant No. 2, stir + precipitate No. 1 swine pepsin; filtrate. ....		0.85					
5 ml. supernatant No. 2 from swine pepsin, added to precipitate No. 1 from bovine pepsin, stir, centrifuge and filter, supernatant. ....						1.60	0.32
Calculated if solubilities additive. ....						1.49	0.29

saturated magnesium sulfate. The solubility determined in this way agreed with that found by stirring the precipitate. This shows that the value is an equilibrium one since the same value is obtained from both sides. The experimental technique was the same as that previously described (6).

A solution of three times recrystallized swine pepsin was treated in the same way. The solubility of the bovine pepsin determined in this manner was found to be slightly less than that of the

swine pepsin but, owing to the small quantity available, this difference could not be considered significant. However, when the saturated solution of the swine pepsin was added to the solid bovine pepsin and the suspension stirred, the total protein and activity of the resulting solution was very nearly equal to the sum of these quantities as determined separately with the two preparations. The solubilities of the two preparations are, therefore, additive which shows that the proteins are chemically different and form mixtures in the solid phase. The results of these experiments are shown in Table III.

The solubility of the swine pepsin is slightly higher than that previously found (12) owing to slightly lower magnesium sulfate concentration and slightly higher temperature.

#### SUMMARY

1. A method has been described for isolating a crystalline protein with high proteolytic activity from bovine gastric juice by means of precipitation with magnesium sulfate and fractionation of the precipitate with acetone and magnesium sulfate.

2. The crystalline protein obtained in this way has the same crystalline form, optical activity, and specific activity, as determined by a number of methods, as does the crystalline protein previously isolated from swine gastric mucosa.

3. The solubility of the two preparations, however, is additive so that they are different although very closely related proteins.

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# THE PROCESS OF PHAGOCYTOSIS

## THE AGREEMENT BETWEEN DIRECT OBSERVATION AND DEDUCTIONS FROM THEORY\*

By EMILY B. H. MUDD AND STUART MUDD

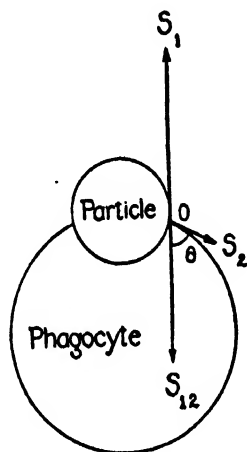
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PLATES 1 AND 2

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The importance of surface forces in phagocytosis has long been recognized. Rhumbler (1) successfully imitated many of the features of phagocytosis by simple physical models, and formulated his conception of its mechanism in terms of surface forces. Tait (2) subsequently treated the mechanism of phagocytosis in terms of the interfacial tensions involved. Fenn (3) recognized the earlier treatments as incomplete, and was the first to work out a satisfactory formulation of the surface forces concerned in phagocytosis. He showed that the same formulation could be derived either from considerations of free surface energy or of interfacial tension. Fenn's treatment has recently been elaborated by Ponder (4).

The surface forces involved in the ingestion of a particle are the interfacial tensions between the three phases in contact. Let Text-fig. 1 be a section through suspending medium, phagocyte, and partially ingested particle. Let  $O$  be a representative point in the line of contact between the three phases; let the vectors  $S_1$ ,  $S_2$ , and  $S_{12}$  be the interfacial tensions,



TEXT-FIG. 1

\* This investigation has been aided by a grant from the Faculty Research Committee of the University of Pennsylvania.



respectively, in the particle-fluid, phagocyte-fluid, and phagocyte-particle interfaces. If  $S_1 > S_{12} + S_2$  the surface of the phagocyte would be drawn completely around the particle and ingestion would occur, provided viscosity or other forces did not interfere with the action of the surface forces. If  $S_{12} > S_1 + S_2$  neither ingestion nor adhesion of particle and phagocyte would occur under the action of surface forces. When  $S_1 < S_{12} + S_2$  and  $S_{12} < S_1 + S_2$ , the surface forces are in equilibrium with the particle in a position of partial ingestion, as shown in the figure; the position taken by the particle at equilibrium is such that  $S_1 = S_{12} + S_2 \cos \theta$ .

Unfortunately the treatment of surface forces in phagocytosis as formulated mathematically by Fenn and Ponder is not susceptible of rigorous experimental test, for the reason that two<sup>1</sup> of the interfacial tensions involved are not measurable. It is possible, however, to determine qualitatively whether or not the behavior of phagocytic cells, as directly observed and as analyzed by experiment, is in agreement with the implications of the mathematical theory.

In the present communication we propose to examine three deductions from the theory of Fenn and Ponder. These are (1) that a quantitative correlation should exist between phagocytosis and the surface properties of the particles ingested; (2) that phagocytosis is essentially a phenomenon of spreading of the phagocyte surface over the surface ingested; and (3) that partial ingestion should occur under certain circumstances.

*Correlation between Phagocytosis and Surface Properties.*—The first obvious deduction from a theory which assigns to surface forces a principal part in phagocytosis is that phagocytosis should be related in some orderly way with the surface properties of the particles phagocytized. This relation has been verified over a very considerable range of experimental conditions (7–14). Various bacteria, erythrocytes, and protein-coated collodion particles have been treated with graded concentrations of the phagocytosis-promoting substances of sera. The electric charge, isoelectric point, wetting properties, and cohesiveness of such series of sensitized particles have been estimated in

<sup>1</sup> It appears possible that at least an upper limit might be assigned to the phagocyte-liquid interfacial tension by application of the method of Harvey (5) or of a modification of that of Cole (6).

independent tests, and the phagocytosis of the particles has been quantitatively determined, using mammalian phagocytes both of the polymorphonuclear and large mononuclear types. A remarkably close correlation between phagocytosis and the surface properties of the particles undergoing ingestion has been regularly found. As the surface properties of the test particles were altered step by step in the series of serum dilutions, phagocytosis was increased in close parallelism. The conclusion drawn from this work is that the phagocytosis-promoting substances of immune sera form on the particles with which they interact a surface deposit upon which phagocytes can spread (12).

In the present communication we shall compare the other deductions from the physical theory with the behavior of phagocytes under direct observation.

### *Experimental Methods*

*Phagocytes.*—Exudative polymorphonuclear leucocytes (7) and large mononuclear phagocytes (macrophages) (13) have been obtained from the peritoneal cavity of rabbits by methods elsewhere described. These were washed in 0.85 per cent NaCl or Ringer's solution and suspended in slightly diluted rabbit serum. In the major part of the work the cells used were samples from the same lots used in quantitative phagocytosis experiments (13, 14). Human polymorphonuclear leucocytes were used in a number of experiments. A platinum loopful of leucocyte suspension, a loopful of the suspension of particles to be phagocytized, and a loopful of specific immune rabbit serum were placed on a carefully cleaned slide, mixed, and a clean cover-slip was gently lowered on top. The edges of the cover-slip were sealed with Salvoline. In such films some leucocytes were freely suspended, some were spread out on the slide, and some on the cover-slip; only rarely was a single cell in contact both with slide and cover-slip. The preparations were put immediately under microscopic observation in a warm-box kept near 37°C.

*Particles Phagocytized.*—Suspensions of washed sheep and washed chicken erythrocytes, *Bacterium typhosum*, *Bacillus subtilis*, and *Monilia albicans* were used. Specific rabbit antisera were prepared for the sensitization of each type of cell. For observation and photography of the bacteria with the bright-field, they were first stained with carbolfuchsin and then washed four to five times. Erythrocytes and monilia were not stained. With the dark-field no staining was necessary.

*Optical Apparatus.*—For transmitted light, Zeiss aplanatic N. A. 1.4 condenser. For dark-field, Zeiss cardioid condenser. Zeiss apochromatic 60 × objective with iris diaphragm. Zeiss 20 × compensating ocular. Zeiss microscope incandescent lamp No. 1 with 165 watt Mazda projection bulb. Zeiss Phoku camera. Hyper-sensitive panchromatic plates. For transmitted light, yellow G filter No. 15.

Exposure time with transmitted light, 3 seconds; with dark-field, 30-60 seconds. Developer D 11 contrast (Eastman). Developed 5 minutes, room temperature. The superficial protoplasm of the phagocyte and the multiform processes and membranes to which it gives rise can be seen more clearly with the cardioid condenser than with any other optical arrangement with which we are familiar.

#### EXPERIMENTAL RESULTS

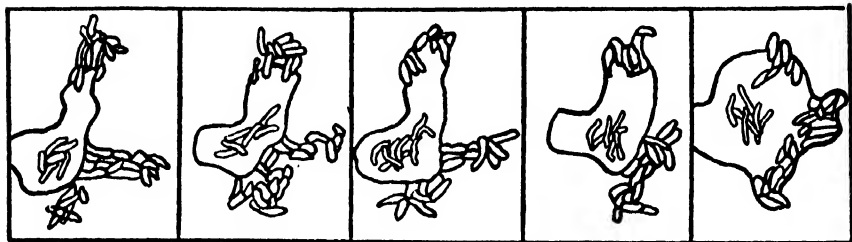
*Phagocytosis a Phenomenon of Spreading.*—It follows both from the mathematical formulation of Fenn (3) and Ponder (4) and from the experimental analysis (7-14) that the capacity of the phagocyte to spread over the surface of the particle undergoing ingestion is a principal factor in determining phagocytosis. Is this deduction in agreement with the process of phagocytosis as directly observed? This question we have examined with especial care. Prediction and observation have been found to be uniformly in agreement.

In mixing the phagocytes, erythrocytes or bacteria and serum as described, many collisions between phagocytes and test particles are brought about. Additional contacts between particle and phagocytes may later be made by the locomotion of the latter. In the absence of sensitizing serum the test particles typically neither stick to one another nor to the phagocytes (Figs. 1 and 2) and the particles are not ingested. In the presence of dilute sensitizing serum, agglutination of the particles and adhesion to the phagocytes may be much in evidence with little complete ingestion occurring (Figs. 5, 6, and 11-14). In the presence of more concentrated sensitizing serum, the test particles adhere to the phagocytes and are drawn into their cytoplasm in great numbers (Figs. 7-10, 29, and 30).

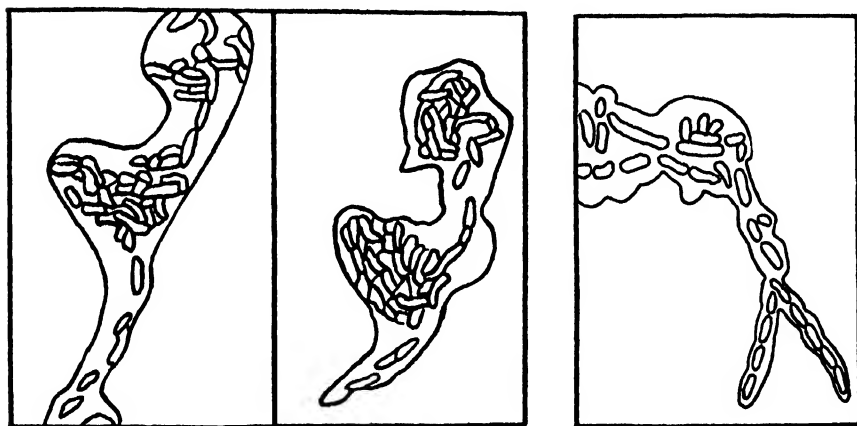
The test particles may be drawn into the phagocytes with comparatively little distortion of the latter (Figs. 29 and 30). Or a process composed of the hyaline superficial protoplasm may flow out over the surface of the particle undergoing phagocytosis (Figs. 3, 4, 15-19, 28, and 34-37). A semidiagrammatic tracing of Figs. 15-19 is shown in Text-fig. 2. Or the spreading of the leucocytes over the sensitized particles may cause marked deformation of the leucocytes (Figs. 20-24 and 25-27). A semidiagrammatic tracing of Figs. 25-27 is shown in Text-fig. 3.

The types of ingestion described of course merge into one another. For instance four chains of sensitized *subtilis* bacilli were seen arranged

in a diamond-shaped figure with a suspended spherical macrophage in their center. When first observed the chains were merely tangent and adherent to the macrophage surface. Gradually the areas of contact between *subtilis* chains and phagocyte surface increased, the adherent tangents becoming arcs of circles, which were slowly drawn into the macrophage protoplasm. The ends of the chains projected for a time beyond the macrophage surface, but these eventually were



TEXT-FIG. 2



TEXT-FIG. 3

drawn in also; in several instances hyaline processes were observed to flow out over the projecting end of the *subtilis* chain as the last step in its ingestion.

For purposes of comparison between observation and deduction from theory the essential point is that in all instances observed the particles were not taken up in vacuoles of the suspending medium; on the contrary *the protoplasm of the phagocytes was in immediate contact with the surface undergoing phagocytosis*. Phagocytosis as observed,

then, is primarily a phenomenon of surface spreading—the spreading of the phagocyte surface over the surface of the object undergoing ingestion. Prediction from theory and from experimental analysis is thus in agreement with observation on this second essential point.

Before leaving this point, however, two possible sources of confusion should be mentioned. Phagocytes of the large mononuclear type are able to form delicate petal-like extensions of their peripheral hyaline protoplasm—the “sheet-like pseudopods” of Smith, Willis, and Lewis (15), the “undulating membranes” of Carrel and Ebeling (16), the “*pseudopodes pétaloïdes*” of Fauré-Fremiet (17) (Figs. 33 and 35). W. H. Lewis (18) has described under the term “pinocytosis,” and shown in moving pictures, the engulfing of tiny vacuoles of the fluid medium by these processes. Should such a vacuole contain a minute particle it would of course be engulfed also. What may have been such an instance has been described by Chambers and Borquist (19). However, although we have seen the phagocytosis of a large number of bacteria and erythrocytes by direct extension of the phagocyte surface over the surface of the particle ingested, we have never observed ingestion in a vacuole. Phagocytosis and pinocytosis we believe to be quite different phenomena.

Another possible source of confusion is the fact that in stained films showing phagocytosis bacteria can often be seen to lie in little vacuoles in the cytoplasm (15, 20). These digestive vacuoles are seen especially about the bacteria which have been ingested for some minutes and have been moved in toward the center of the cell. These vacuoles are a phenomenon not of ingestion but of intracellular digestion.

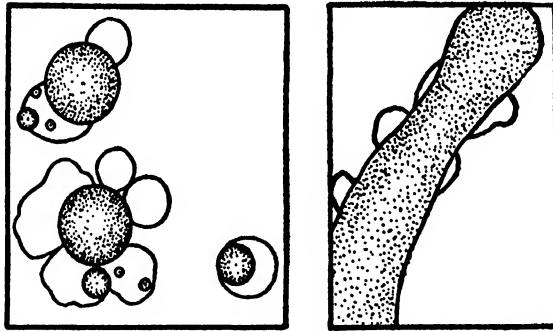
*Partial Ingestion.*—Fenn’s formulation of surface forces in phagocytosis predicts that under certain conditions partial ingestion should occur; this is an important point of departure from the formulations of Rhumbler and Tait. Fenn recognizes two conditions:

(a) The free surface energy is at a minimum when the particle is partially ingested; surface forces are therefore in equilibrium and satisfy the equation  $S_1 = S_{12} + S_2 \cos \theta$ .

(b) Surface forces would tend to bring about complete ingestion, but this is prevented by the resistance to deformation of the phagocyte; surface forces are therefore not in equilibrium but are held in check by viscosity.

The second condition has certainly been realized in our experiments, and to the best of our belief also the first. Figs. 31 and 32 show fields in which macrophages were mixed with an emulsion of light California mineral oil. A tracing of Figs. 31 and 32 is shown in Text-fig. 4. Emulsion droplets of small size are readily and completely ingested by the macrophages. On the larger drops the macrophages spread (Figs. 31 and 32) to positions determined by the balance between surface forces and their own resistance to deformation.

Incidentally it may be mentioned that such small emulsion droplets are very readily ingested by macrophages, but ordinarily not by polymorphonuclear leucocytes. Such a difference cannot be explained by differences in resistance to deformation, since the polymorphonu-



TEXT-FIG. 4. Tracing of Figs. 31 and 32. Macrophages white with black outlines; mineral oil stippled.

clears are on the average more fluid cells than the macrophages. This is evidently an instance in which a difference in the surfaces of the two types of phagocyte is a critical factor in determining phagocytosis. Another such instance was found in the quantitative phagocytosis study;—collodion particles are readily ingested by macrophages but not by polymorphonuclear leucocytes (13).

Partial ingestion with surface forces in equilibrium is more difficult to demonstrate conclusively. When weakly sensitized erythrocytes are mixed with phagocytes partial ingestion often occurs (Figs. 5, 6, and 11–14). Such partially ingested cells are not completely ingested during the time they are kept under observation even though this may be far longer than is required for complete ingestion of more strongly sensitized erythrocytes. It is difficult to believe that the

partial ingestion by such a fluid cell as is shown in Figs. 11-14 could represent anything other than equilibrium under surface forces. Moreover in stained preparations (20) it has very frequently been observed that strongly sensitized bacteria were completely ingested whereas weakly sensitized bacteria under otherwise similar conditions were merely adherent to the surfaces of the phagocytes. Although we realize that such observations fall somewhat short of rigorous proof that the surface forces are in equilibrium, we believe that this is by far the most probable interpretation. The third deduction from theory, namely the occurrence of partial ingestion, is thus likewise in agreement with observation.

*Viscosity.*—L. Loeb (21) has related the ameboid motion of the amebocytes of *Limulus* to "1) changes in consistency in the ectoplasmic layer as well as in the granuloplasm, 2) phenomena of contraction and 3) surface tension changes." Loeb in 1927 sought to carry over these conceptions to the explanation of phagocytosis by mammalian cells, assigning a primary importance to softening of certain parts of the surface layer of the cell in contact with a foreign body. Whether or not such local softening occurs on contact of phagocytes with foreign particles, it is evident that the quantitative correlation which has since been demonstrated between phagocytosis and the surface properties of the particles phagocytized (7-14) is not explained by viscosity changes and is explainable in terms of interfacial tension relations.

The resistance of the protoplasm to deformation is, on the other hand, a modifying factor in phagocytosis which, under certain conditions, may reach critical importance. Fenn (22) for instance found very high temperature coefficients for phagocytosis below 30°C. as compared with those above 30°. He interpreted his data as indicating that below 30° the viscosity of the phagocytes was so high as to become the limiting factor for phagocytosis. Ponder (4) has treated mathematically the retarding influence of viscosity on the rate of phagocytosis. He has shown, moreover, that when ingestion occurs in a moving current such as the blood stream, in which forces may act to dislodge the particle undergoing ingestion from the phagocyte surface, the rate of ingestion as determined by viscosity may become a critical factor.

An average difference in viscosity between phagocytes of the large mononuclear and the polymorphonuclear types has been observed by E. R. and E. L. Clark (23), by Goss (24), and by ourselves (25). The macrophages offer on the average more resistance to deformation than the polymorphonuclears. This difference has been evidenced in the present study in two ways. In the first place the act of ingestion is on the average more quickly accomplished by the polymorphonuclears, and in the second the polymorphonuclears are more readily distorted to all manner of bizarre shapes in spreading over the larger bodies phagocytized (Figs. 25 and 27).

*Unformulated Factors.*—It seems clear then that surface forces are a principal factor in determining ingestion, and that viscosity is an important factor in controlling its rate. It is perhaps worth emphasizing, however, that a complete explanation of the behavior of phagocytes is not afforded by these factors alone. A particle phagocytized under the action of surface forces does not enter a homogeneous liquid, but a system possessing internal organization in high degree. The process which has spread over the particles undergoing phagocytosis is frequently retracted (Figs. 15–26). The protoplasm of the phagocytes possesses elastic properties (Figs. 11–14). The ingested particles are commonly moved in toward the center of the cell. They frequently undergo rapid intracellular digestion. The formation and retraction of pseudopods appears to be the consequence of internal changes within the cell as well as of the tendency of the cell surface to spread upon external surfaces. Reversible changes in viscosity, as evidenced by the appearance and disappearance of Brownian movement, may be seen to occur in local areas within the cell.

#### CONCLUSIONS

The phagocyte, then, is a complex system delicately responsive to internal and external influences. Interfacial tensions, and under certain conditions viscosity, are critical factors in determining the ingestion of particles with which the phagocyte has come into contact. Deductions from the formulation of these factors by Fenn and Ponder are in agreement with observation and with experimental analysis. However, other and still unformulated forces also enter into the behavior of these remarkable cells.



We are indebted to Dr. Balduin Lucké for most of the phagocytes used in this study, and to Dr. Lucké and Dr. Morton McCutcheon for critical consideration of the manuscript.

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## EXPLANATION OF PLATES

## PLATE 1

All figures are unretouched photographs of living phagocytes.

FIGS. 1 and 2. Macrophages (*m*), polymorphonuclear leucocytes (*p*), and sheep erythrocytes. The large macrophage in Fig. 1 contains three ingested oil droplets. No sensitizing immune serum present; little or no agglutination or phagocytosis of erythrocytes.

FIG. 3. A macrophage ingesting a sensitized cell of *Monilia albicans* (*mo*). Note process of macrophage (*ps*) spreading around monilia.

FIG. 4. A macrophage ingesting sensitized sheep erythrocytes. At top erythrocytes being drawn into macrophage. On right a process spreading over two erythrocytes.

FIGS. 5 and 6. Partial ingestion of weakly sensitized sheep erythrocytes by macrophages and polymorphonuclear leucocytes (*p*). The shadows marked (*d*) are dust on the camera lens.

FIG. 7. A cluster of strongly sensitized erythrocytes surrounding and being drawn into a macrophage.

FIGS. 8 and 9. Successive stages in ingestion of a mass of strongly sensitized sheep erythrocytes by a macrophage; the macrophage contains an oil droplet (*o*).

FIG. 10. A macrophage embedded in and ingesting strongly sensitized sheep erythrocytes. The macrophage contains two previously ingested oil droplets.

FIGS. 11-14. Successive stages in migration of polymorphonuclear leucocyte which has partially ingested weakly sensitized sheep erythrocytes. That portion (*a*) of the leucocyte to the left of the figure has partially ingested three erythrocytes which remain adherent to the glass slide; the portion (*b*) of the leucocyte which contains one partially ingested erythrocyte continues to migrate toward the lower right hand corner of the field until the protoplasm of the leucocytes is stretched into a thin filament. While under direct observation the adherent erythrocytes in the upper left hand corner were pulled loose from the glass and the protoplasmic filament contracted like a stretched rubber band.

## PLATE 2

FIGS. 15-19. Successive stages in ingestion of clumps of strongly sensitized typhoid bacilli by a polymorphonuclear leucocyte. In Figs. 15, 16, and 17 a process of the leucocyte spread over a clump of sensitized bacteria shown above the leucocyte. In Figs. 18 and 19 this process contracted, drawing the ingested bacteria toward the center of the cell. In Fig. 19 a second process began to spread over a clump of bacteria to the right of the leucocyte.

FIGS. 20-24. Successive stages in contraction of the process of a polymorphonuclear leucocyte which has spread over a clump of sensitized typhoid bacilli. In Fig. 24 the process has contracted and the bacteria have moved in toward the center of the cell.

FIGS. 25 and 26. A polymorphonuclear leucocyte spread over agglutinated *B. subtilis*. In Fig. 26 the cell is tending to round up and some bacteria have moved toward the center.

FIG. 27. A polymorphonuclear leucocyte spread over a  $\lambda$ -shaped chain of *subtilis* bacilli.

FIG. 28. Two polymorphonuclear leucocytes each filled with monilia cells. A process (*ps*) of the upper leucocyte has just spread around a monilia cell, (*mo*), and the lower leucocyte is spreading over another half-ingested monilia cell.

FIG. 29. A macrophage ingesting *subtilis* bacilli. Two chains of bacilli are adherent to the macrophage surface; the right hand arm of the upper Y-shaped chain has been drawn into the macrophage.

FIG. 30. *Subtilis* bacilli being drawn into a macrophage.

FIG. 31. Macrophages ingesting or spreading on the droplets of an emulsion of mineral oil.

FIG. 32. Macrophages spread out on a peninsula of mineral oil.

FIGS. 33-37. Are dark-field photographs.

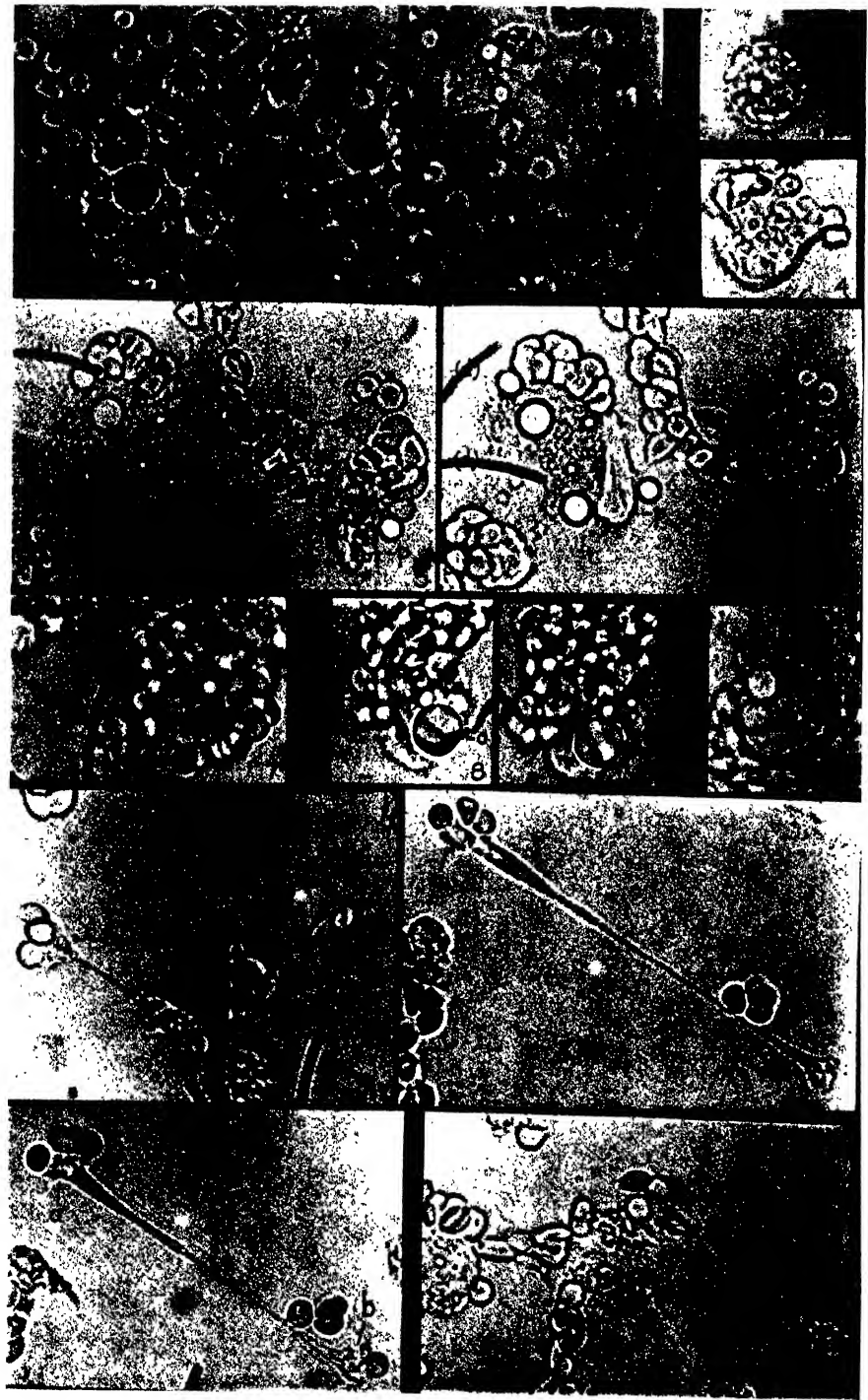
FIG. 33. Macrophage spread out on glass. In order to bring out the detail of the peripheral hyaline protoplasm, the detail of the central granular protoplasm has been lost by overexposure.

FIG. 34. Macrophage extended to a pear-shape by spreading over a *subtilis* chain. The *subtilis* chain (*s*) is the stem of the pear and the vague white around it (*ps*) a process of the macrophage.

FIG. 35. The same macrophage a few minutes later. The cell has thrown out thin "veil-like processes" toward the top of the picture.

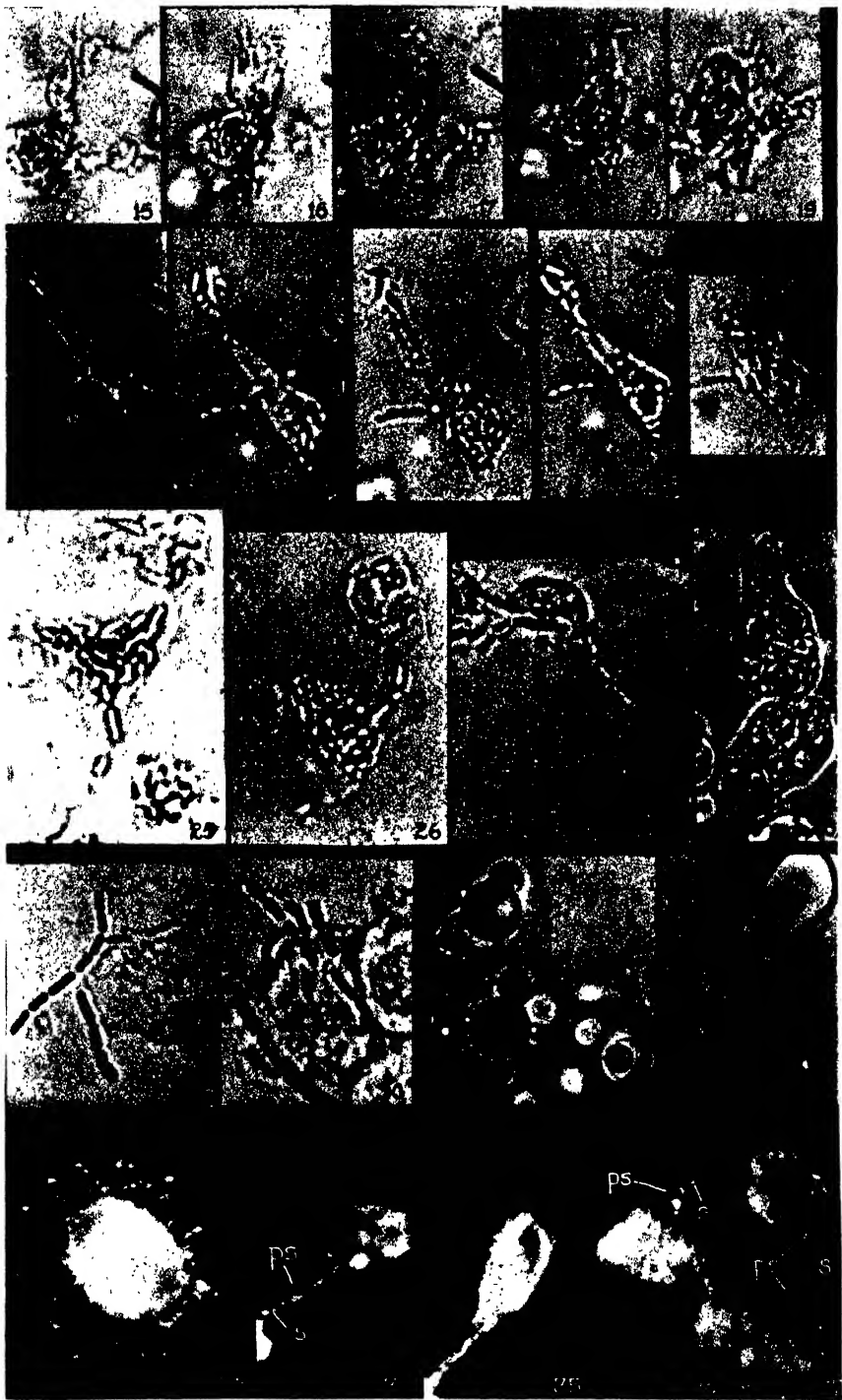
FIG. 36. Macrophage with hyaline protoplasmic process (*ps*) spreading over a *subtilis* chain (*s*). The latter becomes out of focus in the upper right hand corner of the picture.

FIG. 37. Two macrophages with *subtilis* chain (*s*) between them. Each cell has ingested one end of the bacterial chain and has extended a hyaline process (*ps*) on that portion which lies between the cells. The *subtilis* appears as a white chain and the two processes as delicate sheaths with dim outlines separated from the bacteria by dark spaces. The processes from the two cells met and remained approximated for some minutes; both were then withdrawn into their respective cells. One process was observed to extend again out over the *subtilis* chain before the field was lost to view.



(Mudd and Mudd: Process of phagocytosis)





(Mudd and Mudd: Process of phagocytosis)



# THE CHARACTERISTICS OF ULTRAFILTRATES OF PLASMA

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## I

### *On the Establishment of Equilibrium Conditions during the Process of Ultrafiltration*

It has been tacitly assumed by many investigators that the fluid formed by filtration through partially semipermeable membranes will be at diffusion equilibrium with the original fluid. In case one of the indiffusible constituents of the original fluid has an ion in common with a diffusible constituent, the so called Donnan membrane equilibrium would be set up if time adequate for the attainment of equilibrium be allowed. There is an important question as to whether the equilibrium would be set up within the time that the ultrafiltrate is passing through the pores of the filtering membrane. Under experimental conditions when a collodion membrane is used as the ultrafilter, the distance across the membrane is relatively great, that is, of the order of magnitude of tenths of a millimeter, and the time during which the filtered fluid remains on the membrane is so short, that is, a matter of minutes, that it might be supposed that equilibrium conditions would never be set up if they depended upon diffusion equilibrium being reached across the whole membrane. Actually, however, we must conceive of the diffusion equilibrium as being set up across the hypothetical plane bounding the region within which the indiffusible constituents can move freely, and separating that region from the region they cannot enter, presumably because of diminutive pore size. That is to say, looking at the membrane as a collection of pores between solid blocks the membrane equilibrium must be set up across the plane of entrance to the pores. Then, unless the fluid moves so rapidly through the pores that diffusion equilibrium cannot occur, we



should expect to find the membrane equilibrium conditions set up in the fluid of the pores, the limiting factor being the rate of flow of fluid compared with the rate at which diffusion equilibrium is established at a given distance from the point of constant composition.

In order to calculate the distance through which diffusion equilibrium will be substantially complete in a given time, one can use a special application of Fick's diffusion law, which is

$$\frac{dC}{dt} = k \left( \frac{d^2C}{dx^2} \right),$$

when  $C$  is the concentration at any distance  $x$  and time  $t$ , and  $k$  is the diffusion constant.

Andrews and Johnston (1924) have derived the integral for the case of diffusion in a single dimension into a slab, which has the form:

$$\frac{C}{C_1} = 1 - \frac{8}{\pi^2} \left[ e^{-\frac{\pi^2}{4} \cdot \frac{kt}{a^2}} + \frac{1}{9} e^{-\frac{9\pi^2}{4} \cdot \frac{kt}{a^2}} + \frac{1}{25} e^{-\frac{25\pi^2}{4} \cdot \frac{kt}{a^2}} + \dots \right]$$

This series may be simplified by neglecting all terms except the first which are insignificant when diffusion equilibrium is more than 36 per cent complete. The equation then becomes

$$\frac{C}{C_1} = 1 - \frac{8}{\pi^2} \left( e^{-\frac{\pi^2}{4} \cdot \frac{kt}{a^2}} \right)$$

In this expression  $C$  is the average concentration difference from the original plasma concentration over the distance  $x$ ;  $C_1$  is the equilibrium concentration difference due to the Donnan effect;  $k$  is the diffusion constant of the solute under consideration; and the other symbols have their usual meanings. It is assumed that diffusion is in one plane and that the concentration at the inside wall of the membrane is constant and equal to that in the body of the interior fluid from which ultrafiltration is occurring.

The diffusion constant of sodium chloride in water, according to Landolt-Börnstein, is 0.94 cm.<sup>2</sup> per day. Using this value for  $k$  one can calculate the ratio  $\frac{C}{C_1}$  at various values of  $t$  and  $a$ . In Fig. 1 are plotted the values of  $a$  for the times at which equilibration is 95 per cent complete. This proportion may be taken because the difference

between plasma concentration and final ultrafiltrate concentration is of the order of magnitude of 20 mg. per cent chloride, and 95 per cent of this difference represents the limit of experimental accuracy in determination. It is to be seen that at 1 second such an approach to equilibrium would be established at a distance of 0.03 mm. from the plane of separation. In the same figure is shown the maximum rate of flow through the pores of the membrane, calculated from the data of

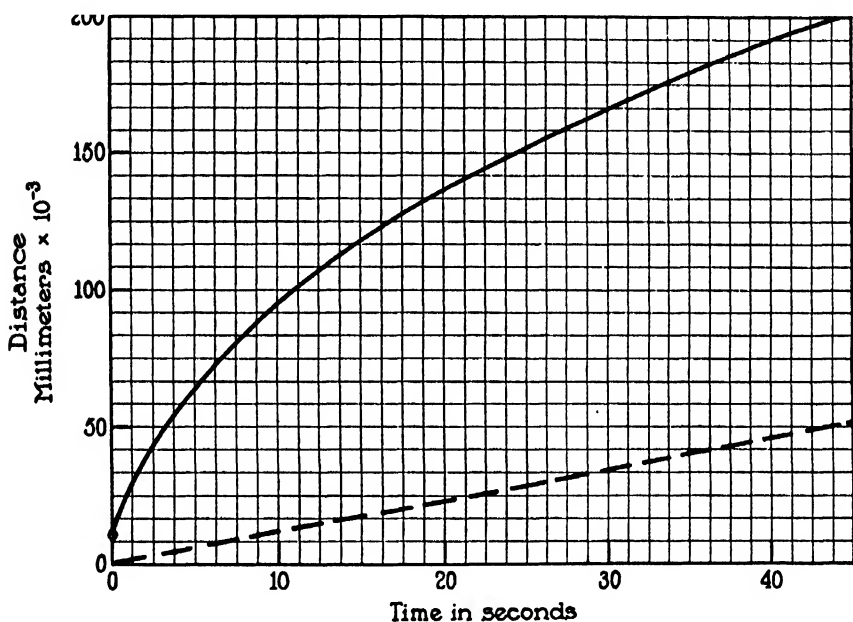


FIG. 1. The distance over which diffusion equilibrium is substantially complete in relation to time. The solid line shows the course of the diffusion equilibrium distance with time. The lower broken line shows the distance over which fluid is passing in the pores of the membrane according to the calculations in the text.

Hitchcock (1926) for the dimensions and the number of pores per unit area of membrane, and our own figures for the rate of flow across the membrane. According to Hitchcock the minimum pore radius in such membranes is  $0.3 \times 10^{-6}$  cm. and the minimum frequency of distribution is  $7 \times 10^{10}$  per  $\text{cm}^2$ . According to our observations the maximum rate of filtration was  $2.27 \times 10^{-6}$  cm. per second, assuming that the whole surface was permeable. Taking Hitchcock's figures, the minimum pore area is  $0.198 \text{ cm}^2$  per  $\text{cm}^2$  of membrane surface.

Using this figure it appears that the maximum velocity of flow through the pores of the membrane is  $1.14 \times 10^{-4}$  cm. per second.

This figure represents the maximum velocity, according to the data at hand, which the fluid flowing through the pores of the collodion membrane in our ultrafiltration experiments would have. It is apparent from the graph that at this rate of flow equilibrium will certainly be substantially complete while the fluid is moving through the pores. It is also obvious that with membranes having a thickness of 0.2 mm. diffusion equilibrium would be complete in the time available even if that equilibrium had to occur across the whole membrane. Thus it is apparent that one should expect to find the membrane equilibrium set up even during the dynamic process of ultrafiltration, by virtue of the fact that the mechanical flow of fluid is relatively very slow compared with the rapidity with which diffusion equilibrium is set up over short distances. It seems obvious from our measurements and calculations that at least until the rate of flow through the pores reaches a speed of  $5 \times 10^{-2}$  mm. per second, which is 50 times the speed we calculate to occur, diffusion equilibrium should be established.

## II

### *The Exact Measurement of the Concentration of Electrolytes in Ultrafiltrates*

There are hundreds of papers upon the subject of the composition of ultrafiltrates of plasma, but in none of them is there any clear evidence as to whether or not the distribution of ions is influenced by the membrane equilibrium. For the most part investigators have been concerned with the question as to whether one or another constituent of plasma is freely diffusible. The criterion has usually been the existence of identical concentrations of the constituent in the plasma and the ultrafiltrate. Obviously this cannot be the correct criterion if the proteins influence the ionic activity or if the Donnan effect occurs. In most instances, furthermore, investigators have neglected to calculate their results on the basis of the water content of the fluid rather than the volume. Inasmuch as in the case of plasma an error of about 8 per cent results from such neglect, it is not surprising that the conclusions from such observations should be inadequate. Neu-

hausen and Pincus (1923) found that collodion membrane ultrafiltrates of plasma showed nearly the same concentration of sodium chloride as the plasma from which they were derived. In their experiments there was a random difference which they did not attempt to interpret or analyze. Several other investigators have made similar observations. Particularly in view of the fact that physiologists are assuming that certain ultrafiltrates or dialysates of plasma such as ascitic fluid and other edema fluids should show differences in the concentration of ions predicted by the Donnan membrane equilibrium, it seems desirable to make an exact study of the ultrafiltration process itself with sufficiently careful analytical methods, in order to be able to establish the existence or non-existence of such a membrane equilibrium.

In such a study it becomes necessary in the first place to determine with adequate methods what differences if any exist between the concentration of various ions in the plasma and ultrafiltrates from it, and to see whether changes in the base bound by protein, which would be anticipated to affect the membrane equilibrium distribution ratio, do actually alter it. For this study we have chosen the sodium, potassium, and chloride ions for measurement. Some of the finer points in analytical technique, which may be disregarded when less exacting results are wanted, have had to be closely attended to in this investigation.

#### (a) *Methods*

Collodion sacs for ultrafiltration were prepared from a solution of 10 gm. of dry pyroxylin in 100 cc. of equal parts of alcohol and ether, to which 5 per cent of glycerin was added. The collodion solution was poured into chemically clean 25 x 200 mm. Pyrex test-tubes. The excess was drained by inverting during slow rotation. After 5 minutes rotation the casting was repeated and the test-tube was set up inverted on a piece of dry filter paper for a period of  $\frac{1}{2}$  hour, after which the membrane was removed from the test-tube and immersed in distilled water saturated with chloroform, where it was kept at refrigerator temperature until use. A large number of membranes were made at one time and used for a number of experiments. They were tested as to their impermeability for protein, and practically all membranes made by this method were found to be impermeable. The sacs were blotted dry with clean filter paper before use. They were then rinsed three or four times with plasma and tied securely to perforated rubber stoppers. They were filled through the holes of the stopper and were then placed in a glass jacket

fitted with two side arms for flushing with air of proper composition as noted below. Pressure was applied over the fluid in the sac and maintained constant by the use of a large air pressure bottle, using a mercury manometer to measure pressure. The air used in the pressure bottle and over the fluid in the sac, as well as in the jacket, was obtained from a large reservoir maintained at the  $\text{CO}_2$  tension to which the plasma had been equilibrated and possessing the same vapor tension as the plasma, by having been passed through 0.9 per cent sodium chloride. In this way possible errors due to evaporation and changing  $\text{CO}_2$  tension were eliminated. The fluid for analysis was measured by weight inasmuch as it is impossible to calibrate a pipette to deliver accurate volumes of fluids of varying surface tension and viscosity, such as are possessed by plasma and ultrafiltrate. This is a very important point, and failure to take it into account leads to serious error. It is not necessary to weigh each sample of a given fluid if the same pipette is used for the successive samples, since a single weighing will obviously determine what the pipette will deliver of a given fluid. In practise it is therefore only necessary to weigh one quantity of every specimen, and this was done with the specimen used for determining total solids and water.

The chlorides were determined by the digestion method of Van Slyke (1923) during the major part of this investigation. In our first twenty experiments we attempted to use the Whitehorn method but found that, although with certain samples of plasma results obtained agreed with those obtained by the theoretically more accurate digestion method, there was usually a difference showing a larger quantity of chloride in plasma by the Whitehorn (1921) method. This difference we believe to be due to the fact that in making the dilution accompanied by protein precipitation the volume of the protein precipitate is neglected. It seems that this introduces a systematic error of at least 3 per cent, which would enter in only in the case of the protein-containing fluid and would therefore completely invalidate the method for comparison of plasma with its ultrafiltrate. We have found the digestion method to be reliable under all circumstances under which we have tested it. The sodium was determined by the uranyl zinc acetate method of Kolthoff and Barber (1928). For the determination of sodium the plasma samples (1 cc.) were ashed by repeated evaporation to dryness with 1 cc. concentrated nitric acid to which 3 or 4 drops of superoxol had been added. Usually two or three evaporations were necessary. The analysis of sodium has been one of the major difficulties in the approach to the problem under investigation, and we have therefore carried out a rather careful study of the analytical accuracy of this method. The results which we have to present depend for their validity upon the accuracy with which the ions in question can be determined, and since the method is relatively untried, we carried out 50 consecutive analyses of the same sample of plasma to estimate the probable error of the method. The results are given in Table I. In the sample of plasma containing 338.9 mg. per cent sodium the maximum deviation was 1.70 per cent. The mean deviation was 0.515 per cent and the probable error of the mean 0.059 per cent. It is to be seen that a very high degree of accuracy is possible. Potassium was determined by the colorimetric technique

of Breh and Gaebler (1930), using the sodium-silver-cobaltinitrite method. In analysis of known solutions we have found this method to be fairly accurate, although not so accurate as the sodium method. Potassium has been found to behave somewhat differently from sodium in the ultrafiltration process, and as high a degree of accuracy is not necessary in order to make sure of this difference.

TABLE I  
*Analysis of Sodium Method*

50 consecutive analyses of 1 cc. samples of one specimen of blood					
Sodium	Deviation from mean	Sodium	Deviation from mean	Sodium	Deviation from mean
<i>Mg. per 100 gm. H<sub>2</sub>O</i>	<i>per cent</i>	<i>Mg. per 100 gm. H<sub>2</sub>O</i>	<i>per cent</i>	<i>Mg. per 100 gm. H<sub>2</sub>O</i>	<i>per cent</i>
340.1	0.35	337.7	0.35	340.3	0.41
340.7	0.52	336.8	0.61	339.8	0.26
336.7	0.64	336.7	0.67	338.2	0.20
338.6	0.09	342.3	1.00	338.2	0.20
339.6	0.20	336.4	0.73	341.8	0.84
335.6	0.96	340.0	0.32	341.2	0.67
341.2	0.67	338.2	0.20	335.7	0.93
335.9	0.87	335.0	1.13	337.4	0.44
341.3	0.70	340.3	0.41	340.5	0.46
339.5	0.17	340.0	0.32	340.3	0.41
340.3	0.41	338.8	0.03	342.0	0.90
338.3	0.17	336.5	0.70	342.2	0.96
336.5	0.74	338.8	0.03	341.9	0.87
340.8	0.55	338.2	0.20	337.0	0.55
341.2	0.67	337.1	0.52	337.7	0.35
338.9	0.00	340.0	0.32	339.5	0.17
332.9	1.70	338.3	0.20		

Mean of all determinations = 338.9 mg. per cent.

Mean deviation =  $\pm 0.515$  per cent.

Standard deviation =  $\sigma = \frac{\sqrt{\sum d^2}}{n} = 0.6139$  per cent.

Probable error of the mean P.E.<sub>m</sub> =  $\frac{0.6745\sigma}{\sqrt{50}} = 0.0585$  per cent.

Sodium, chloride, and potassium were determined in duplicate in each instance. The hydrogen ion concentration of the plasma was measured with the quinhydrone electrode using a potentiometer and galvanometer of adequate accuracy. The carbon dioxide content of the plasma was determined by the method of Van Slyke and Neill (1924). In equilibrating the plasma with definite tensions of CO<sub>2</sub>, the CO<sub>2</sub>-containing air was stored in a Douglas bag and its composition was determined by the Haldane method.

Ultrafiltration was carried on at a pressure of 100 mm. of mercury and the rate of filtration was from 2 to 4 cc. per hour through approximately 50 cm<sup>2</sup>. area of membrane. After establishing the pressure at the commencement of filtration, the surrounding jacket was removed and the first 0.5 cc. of filtrate was absorbed from the wall of the collodion sac by dry clean filter paper, in order to eliminate the error of dilution due to the water retained in the pores of the membrane.

*(b) The Concentration of Sodium and Chloride in Ultrafiltrate and Plasma*

The first question to be decided is as to whether the concentration of the several ions under investigation is identical in an ultrafiltrate and in the plasma from which it came. In Table II are shown the data

TABLE II

	Proteins	Solids	[ $\text{Cl}^-$ ]	[ $\text{Na}^+$ ]
	per cent	per cent	mm. per kg. H <sub>2</sub> O	mm. per kg. H <sub>2</sub> O
Plasma.....	5.9	6.48	128.6	151.1
Residue.....	7.4	7.96	126.6	157.9
Mean plasma.....			127.6	154.9
Ultrafiltrate.....	0	1.07	132.7	141.8

$$r_{\text{chloride}} = \frac{[\text{Cl}^-]_p}{[\text{Cl}^-]_u} = 0.963$$

$$r_{\text{sodium}} = \frac{[\text{Na}^+]_u}{[\text{Na}^+]_p} = 0.916$$

of a typical experiment. It is to be noted that the concentration of sodium and chloride in the water of the ultrafiltrate is different from that of the original plasma, while the concentration in the residue left in the collodion sac is likewise different from the original. The ultrafiltrate was being formed from a plasma of changing composition; presumably, therefore, the concentration of the ultrafiltrate was not constant through the whole process, but was gradually changing. It is therefore the composition of an average ultrafiltrate that we measure, and it is consequently improper to compare it with the concentration in the original plasma; rather one must compare it with the average concentration of the fluid over the time during which it was filtered. Taking the mean of the residue and the original plasma, and comparing

with the ultrafiltrate, which is an average, the ratio one obtains is the true concentration ratio. In the case of sodium one finds the concentration is less in the ultrafiltrate than in the plasma. In the case of chloride one finds more in the ultrafiltrate than in the plasma from which it came.

It is important to note that this inverse behavior of sodium and chloride would be predicted if their distribution were determined by the forces entering into a membrane equilibrium when an indiffusible anion is present. This may be taken as suggestive but not conclusive evidence that the membrane effect produces the concentration differences.

TABLE III

*Concentration Ratio for Sodium in Consecutive Ultrafiltrations of Several Samples of Dog's Plasma Equilibrated with Air Containing 6 per cent CO<sub>2</sub>*

0.952	0.900*	0.918	0.911
0.914	0.913*	0.955	0.951
0.940	0.908†	0.929	0.914
0.902	0.919*	0.978	0.916
0.930†			

Mean = 0.926

$\sigma$  =  $\pm 0.021$

P.E.<sub>m</sub> =  $\pm 0.004$

\* Average of three complete ultrafiltrations on one sample of plasma.

† Average of two such complete runs on one plasma.

In order to determine whether the difference between the concentration of sodium in the water of the plasma and in the water of the ultrafiltrate is a significant one in relation to the possibilities for analytical error in the method, it is necessary to study a series of observations. In Table III are shown the results with seventeen samples of plasma representing twenty-three complete ultrafiltration experiments. The mean value for the concentration ratio for sodium in ultrafiltrate as compared with the plasma from which it was derived is 0.926. The difference between this and the ratio 1.000, which would exist if there were an equivalence in concentration of the two, is 0.074. This value is more than three times the standard deviation and is nearly twenty



times the probable error of the mean of this series. There is obviously a significant difference. Comparing the probable error of the mean for the concentration ratio with the probable error of the mean of a series of determinations of a single sample of plasma, as shown in Table I, it is to be noted that the probable error of the former is about seven times the probable error of the mean in the latter. This is due to the fact that in the calculation of a "concentration ratio" analytical values in three separate fluids must be dealt with. The random variations due to the inherent inaccuracies of the technique therefore enter in three times, and although they may cancel each other out—and usually do—they will on occasion be in the same direction and therefore cumulative.

In the case of chloride the concentration ratio  $r$  has been found to have an average value of 0.969 for plasma equilibrated with CO<sub>2</sub>-containing air, as is shown in Table V. There is thus a difference of 0.031 between the ratio observed and that which would occur if the concentration of chloride were identical in plasma and ultrafiltrate. The standard deviation in this series, which represents twenty-three complete ultrafiltrations on thirteen samples of plasma, is 0.009 and the probable error of the mean is 0.0016. One can state with assurance that the distribution of chloride in plasma and ultrafiltrate is unequal and that therefore some factor such as the membrane effect must enter into the process.

*(c) The Influence of Changing the Amount of Base Bound by Protein*

In the course of these experiments it was observed that there was a correlation between the bicarbonate content of the plasma and the sodium and chloride concentration ratios. We therefore compared the concentration ratios for each sample of plasma at different hydrogen ion concentrations produced by equilibrating portions of the plasma with differing tensions of CO<sub>2</sub> in air. In Table IV are presented the analytical results of one such complete experiment. It is apparent that raising the pH by diminishing the bicarbonate increases the difference between the concentrations in ultrafiltrate and plasma. That is to say, at the higher pH there is more chloride and less sodium ultrafiltered. In this connection it should perhaps be noted again that the pertinent plasma concentration is the average between the original and the residue.

TABLE IV  
*Influence of Changes in the pH upon the Distribution Ratio in Ultrafiltration*

	Original plasma	pH 6.95		pH 7.75		pH 6.95		pH 7.75		Average ratio for subexperiments	
		Residue	Ultrafiltrate	Distribution ratio	Residue	Ultrafiltrate	Distribution ratio	Residue	Ultrafiltrate		
(a) pH 6.95	422.6	390.0	421.7	0.964	408.6	423.9	0.980	385.5	424.7	0.965	Chloride
(b) pH 7.75	422.0	378.5	432.5	0.926	373.7	429.8	0.926	371.4	430.0	0.925	mg./100 gm. H <sub>2</sub> O
(a) pH 6.95	350.0	360.2	332.3	0.936	367.0	332.6	0.928	364.1	330.7	0.930	Sodium
(b) pH 7.75	349.0	365.2	329.2	0.922	366.3	327.2	0.915	366.1	328.2	0.919	mg./100 gm. H <sub>2</sub> O

Bicarbonate content of plasma at pH 6.95 was 15.6 millimols; in the plasma at pH 7.75 it was 10.5 millimols. The correction has been made for CO<sub>2</sub> in solution.

TABLE V

*Concentration Ratio for Cl and the Change with Alterations in pH*

Experiment No.	$r$ (pH about 7.00)	$r$ (pH about 7.70)	$\Delta r$
1	0.958	0.935	-0.023
2	0.973	0.961	-0.012
3	0.972	0.963	-0.009
4	0.964	0.943	-0.021
5	0.962	0.953	-0.009
6	0.960	0.939	-0.021
7	0.967	0.938	-0.029
8	0.971	0.940	-0.031
9	0.992*	0.951*	-0.041
10	0.976*	0.957*	-0.019
11	0.972†	0.930†	-0.042
12	0.962†	0.944†	-0.018
13	0.965*	0.925*	-0.040
Mean	0.969	0.944	-0.024
$\sigma$	$\pm 0.0087$	$\pm 0.0116$	$\pm 0.0118$
P.E. <sub>m</sub>	$\pm 0.0016$	$\pm 0.0022$	$\pm 0.0022$

\* Average of three complete experiments on the same plasma.

† Average of two such experiments.

TABLE VI

*Change in Concentration Ratio for Sodium with Alteration of Hydrogen Ion Concentration of Plasma*

Series 1		Series 2	
$\Delta \text{pH}$	$\Delta r$	$\Delta \text{pH}$	$\Delta r$
+0.67	-0.014*	†	-0.021
+0.61	-0.021*	†	-0.061
+0.80	-0.023‡	†	-0.068
+0.95	-0.004‡	†	-0.068
+0.85	-0.011*	†	-0.038
Mean	-0.015		-0.051
$\sigma$	$\pm 0.006$		$\pm 0.018$
P.E. <sub>m</sub>	$\pm 0.002$		$\pm 0.005$

\* Figure represents the average of three complete runs on one sample of plasma.

† pH was not measured but CO<sub>2</sub> content determinations indicated  $\Delta \text{pH}$  approximately +0.90.

‡ Figure represents average of two complete runs.

In order to be sure that random variations are not so large as to destroy the significance of the differences we have consistently observed to be as indicated above, it is necessary to make a statistical analysis of a comparable series. Table V shows the data of a number of similar experiments so analyzed. In the last column is shown the change in concentration ratio with the change in pH from 7.00 to 7.70. The average  $\Delta r$  is  $-0.024$ , and the probable error of the mean is only  $0.002$ . All values of  $\Delta r$  have the same sign, and there seems to be no reasonable room for doubt that the pH determines the value of  $r$ . Increasing the alkalinity of the plasma, that is, increasing the base bound by protein, causes more chloride to appear in the ultrafiltrate, and *vice versa*.

In a similar way the data for all comparable observations on sodium are presented and analyzed in Table VI. Here again one invariably finds that  $r$  diminishes with increases in pH. The regularity of the difference is not so great as with chloride, probably because of the greater inaccuracies of the sodium method, but in spite of that fact there is obviously a significant change in the concentration ratio for sodium with a change in hydrogen ion concentration.

(d) *The Behavior of Potassium in Ultrafiltration from Plasma*

The concentration ratio for potassium  $r = \frac{[K_u]}{[K_p]}$  has been found to be  $0.901$  when the plasma is about pH  $7.00$ . When the pH is increased to around  $7.70$  the concentration ratio has been found to fall to  $0.816$ , as indicated in Table VII. Thus in the case of potassium as well as the other ions the hydrogen ion concentration determines the concentration ratio.

In Table VIII are presented for comparison the average concentration ratios for sodium, chloride, and potassium in all comparable experiments. The great difference in concentration ratios for these three ions is unmistakable. If the activity coefficient of each of the ions were the same, and all of the distribution differences were due to the membrane equilibrium phenomenon, the concentration ratios should be identical for all ions. Obviously, then, either the activity coefficients are different for the several ions or the membrane equilibrium effect of indiffusible ions is not the sole factor in bringing about

the distribution differences. The simplest explanation of these findings is probably to be found by making the plausible assumption that the activity coefficients are different. If one were to assume that the activity coefficient of the chloride ion in plasma is the standard for comparison for the other ions, then the sodium ion activity is less than the chloride, while the potassium ion activity is still lower than the sodium.

TABLE VII

*Concentration Ratios for Potassium and the Change with Alteration in pH*

Experiment No.	(pH about 7.00)	(pH about 7.70)	$\Delta r$
14	0.905*	0.845*	-0.060
15	0.897†	0.809†	-0.088
16	0.900†	0.804†	-0.096

\* Average of three complete experiments on one sample of plasma.

† Average of two experiments.

TABLE VIII

*Comparison of Average Concentration Ratios for Cl, Na, and K in all Experiments*

	(pH about 7.00)	(pH about 7.70)
Cl.....	0.969	0.942
Na.....	0.928	0.913
K.....	0.901	0.816

(e) *The Calculation of the Magnitude of the Membrane Equilibrium Effect*

It is not certain that the concentration changes produced in the ultrafiltrate by altering the amount of base bound by protein in the plasma are connected with the membrane equilibrium phenomenon. It is altogether possible that other factors might operate to bring about the same effect, and it is therefore of interest to see how closely one can account quantitatively for the observed phenomena by calculations from known data, using the assumption that the distribution of sodium and chloride on the two sides of the ultrafilter membrane is actually determined by the Donnan membrane equilibrium.

In order to calculate the magnitude of the Donnan distribution ratio one must know accurately the amount of base bound by protein, which most investigators have not attempted. It is possible, however, to calculate more simply and accurately the difference in the ratio  $r$  which would be expected when the pH of the plasma is altered by a change in the  $\text{CO}_2$  tension with which it is equilibrated. From this difference, as the derivation below indicates, can be calculated the change in the base bound by protein which presumably caused it. The change in the base bound by protein was experimentally measured in these experiments by determination of the differences in the  $\bar{\text{HCO}}_3$  content. This is, of course, also a measure of the change in the total concentration of diffusible anions in the plasma, since the bicarbonate is the only diffusible anion that appreciably alters with a change in  $\text{CO}_2$  tension. Thus by comparing the calculated with the observed values for the change in concentration of diffusible anion, or conversely in base bound by protein, one can test whether the membrane equilibrium theory satisfactorily accounts for the observed distribution ratios.

Let  $r_a$  = distribution ratio in the more acid condition.

$r_b$  = distribution ratio in the more basic condition.

$[\bar{\text{Cl}}_{pa}]$  = average concentration of chloride in millimols in the plasma during ultrafiltration at the higher  $\text{CO}_2$  tension.

$[\bar{\text{Cl}}_{ua}]$  = Concentration of chloride in the ultrafiltrate at the higher  $\text{CO}_2$  tension.

$[\bar{A}_{pa}]$  = Concentration of all diffusible anions in the plasma at the higher  $\text{CO}_2$  tension.

$[\bar{A}_{ua}]$  = Concentration of all diffusible anions in the ultrafiltrate at the higher  $\text{CO}_2$  tension.

Subscript  $pb$  indicates concentration in the plasma during ultrafiltration at the lower  $\text{CO}_2$  tension and higher alkalinity.

Subscript  $ub$  indicates concentration in the ultrafiltrate at the higher alkalinity.

From the classical Donnan equation:

$$r_a = \frac{[\bar{\text{Cl}}_{pa}]}{[\bar{\text{Cl}}_{ua}]} = \frac{[\bar{A}_{pa}]}{[\bar{A}_{ua}]} \quad (1),$$

and

$$r_b = \frac{[\bar{\text{Cl}}_{pb}]}{[\bar{\text{Cl}}_{ub}]} = \frac{[\bar{A}_{pb}]}{[\bar{A}_{ub}]} \quad (2).$$

From the law of electrostatic neutrality and the fact that  $\text{Na}^+$  is the quantitatively important cation one can state that:

$$[\text{Na}_{ua}^+] = [\bar{A}_{ua}] \quad (3),$$

and

$$[\text{Na}_{ub}^+] = [\bar{A}_{ub}] \quad (4).$$

By substitution in (1) and (2)

$$[\bar{A}_{pa}] = r_a \times [\text{Na}_{ua}^+] \quad (5),$$

and

$$[\bar{A}_{pb}] = r_b \times [\text{Na}_{ub}^+] \quad (6).$$

For convenience one can define:

$$\Delta[\bar{A}_p] = [\bar{A}_{pa}] - [\bar{A}_{pb}] \quad (7).$$

Since with a change in  $\text{CO}_2$  tension the only diffusible anion whose concentration changes is the bicarbonate ion,

$$\Delta[\bar{A}_p] = \Delta[\bar{\text{HCO}}_3] \quad (8),$$

where  $\Delta[\bar{\text{HCO}}_3]$  is the difference in millimols between the bicarbonate content of the plasma in the more acid and the more basic conditions.

If one substitutes the experimentally determined values given in Table IV in equations (5) and (6) one finds:

$$[\bar{A}_{pa}] = 0.965 \times \frac{331.2}{2.3} = 138.9$$

and

$$[\bar{A}_{pb}] = 0.925 \times \frac{328.2}{2.3} = 131.9.$$

Therefore from equation (7)  $\Delta[\bar{A}_p] = 7.0$ . The measured value of  $\Delta[\bar{\text{HCO}}_3]$ , which should equal  $\Delta[\bar{A}_p]$ , is 5.1. Computation for thirteen such complete ultrafiltrations shows that the calculated average value for  $\Delta[\bar{A}_p] = 5.4$  mm., while the experimentally determined quantity  $\Delta[\bar{\text{HCO}}_3] = 4.8$  mm. The agreement between the calculated and the

observed values is sufficiently close to permit the conclusion that the membrane equilibrium theory will account quantitatively for the facts observed.

(f) *The Influence of Ionic Activity upon the Distribution Ratios*

It is interesting to consider the possible reason for the differing distribution ratios for the several ions. It seems most likely that these differences are due to differing ionic activities for the several ion species. If the assumptions are made that the activity of the chloride ion is not influenced by the presence of the protein in plasma and that the sodium ion activity in the ultrafiltrate is equivalent to its activity in other solutions of similar ionic strength, it is possible to calculate the activity coefficient of the sodium ion in the plasma from the observed distribution ratios. Stadie and Sunderman (1931) have shown that the chloride ion activity is not lowered in hemoglobin solutions, whereas the activity of sodium in hemoglobin combination is markedly lowered. It is furthermore possible to calculate the difference in ionic activity with a change in the hydrogen ion concentration of the plasma. For the more acid condition one may write

$$r_a = \frac{[\bar{\text{Cl}}_{pa}]}{[\bar{\text{Cl}}_{ua}]} = \frac{\alpha_{ua} [\text{Na}_{ua}^+]}{\alpha_{pa} [\text{Na}_{pa}^+]}$$

and

$$\frac{\alpha_{pa}}{\alpha_{ua}} = \frac{1}{r_a} \times \frac{[\text{Na}_{ua}^+]}{[\text{Na}_{pa}^+]}$$

For the more basic condition

$$\frac{\alpha_{pb}}{\alpha_{ub}} = \frac{1}{r_b} \times \frac{[\text{Na}_{ub}^+]}{[\text{Na}_{pb}^+]}$$

where  $\alpha_{ua}$  represents the activity coefficient for the sodium ion in the ultrafiltrate,  $\alpha_{pa}$  the same in the plasma, and other symbols as before. In our observations at pH 7.0 summarized in Table VIII the activity ratio  $\frac{\alpha_{pa}}{\alpha_{ua}}$  for sodium is equal to 0.958, which implies that the activity of sodium in the plasma is depressed somewhat more than 4 per cent



by the presence of the protein. Similarly in the case of potassium one calculates that at pH 7.0 the activity ratio is 0.929, while at pH 7.7 it is only 0.866. Thus it is apparent that the values for the distribution ratios of sodium and potassium lower than the value for chloride can be interpreted as due to the depression of the activity coefficient for the cations by the protein in the plasma. It is impossible from the data at hand to be certain as to the exact magnitude of the effect, but it appears to be very probable that in the altered distribution ratio for cations from that for chloride, one is dealing with an effect of the altered activity coefficient of cations by proteins on the alkaline side of their isoelectric points.

Greene and Power (1931) have preferred to treat their data on dialysis of plasma from the point of view of the degree of dissociation of alkali proteinates. Their experimental findings concerning the percentage inactivation of the several cations by the influence of the protein in the plasma agree with the results presented here.

#### *(g) The Biological Significance of These Studies*

It seems apparent from the results here presented that in order to judge as to whether a biological fluid is or is not an ultrafiltrate of blood plasma it is necessary to take into account the fact that ultrafiltrates apparently come into membrane equilibrium concentration with the fluids from which they are formed. Furthermore, the membrane equilibrium ratio is very considerably altered by changes in the hydrogen ion concentration of plasma over physiological ranges. Consequently ultrafiltrations must be made at the hydrogen ion concentration and carbon dioxide tension existing in the organism before results of such ultrafiltrations can be interpreted in connection with this problem.

These results seem to stress further the importance of more careful study of the activity of the several ions in biological systems, and show the inadequacy of comparing analytical concentrations directly as a test for determining whether or not a fluid is a simple ultrafiltrate.

#### SUMMARY

1. Calculations from the Fick diffusion law are shown to predict that membrane equilibria should be established during the course of ultrafiltration.

2. It is shown that the chloride ion is more concentrated and the sodium ion less concentrated in the ultrafiltrate than in the plasma from which the ultrafiltrate was derived.

3. It has been found that by increasing the base bound by protein through a reduction in the bicarbonate content the difference between the plasma concentration and the ultrafiltrate concentration for the several ions studied increases.

4. Calculations from the Donnan equation as to the magnitude of the change in base bound by protein at differing hydrogen ion concentrations are in substantial agreement with the observed values, thus rendering it probable that the membrane equilibrium effect is responsible for the change in distribution ratios observed.

5. It is pointed out that the observed difference in the distribution ratio of cations from that of the chloride anion is probably to be explained by the influence of protein in lowering the activity coefficient of cations when on the alkaline side of the isoelectric point.

6. It is pointed out that account must be taken of these observations in any consideration of the rôle of ultrafiltration in the production of any secretion or body fluid.

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# THE EFFECT OF CYANIDE AND OF VARIATION IN ALKALINITY ON THE OXIDATION-REDUCTION POTENTIAL OF THE HEMOGLOBIN-METHEMOGLOBIN SYSTEM

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Since Ray Lankester (1869) studied the effects of cyanogen upon hemoglobin, numerous divergent reports, relative to compounds of cyanide with the blood pigment have appeared in the literature. These reports may now be interpreted in light of recent investigations on the physical chemistry of the hemoglobin molecule. For instance, that Hoppe-Seyler (1868) obtained a crystalline reaction product of hemoglobin and cyanide we neither doubt, nor explain as did Gamgee (1898) on a basis of inadequate separation. Hemoglobin has many basic groups functioning throughout the pH range over which cyanion is bound and there are reasons for assuming the existence of ferrous and ferric hemoglobin cyanides of varying composition; cyanide being linked to one or more of the basic groups of the globin radicle, as any protein may combine with acidic radicles.

In this study we are more interested in the nature of cyanide linkages with the prosthetic groups of hemoglobin derivatives. The conclusions of Warburg (1923), in which he ascribes the rôle of respiratory catalyst to the hematin-hemochromogen system; the wide distribution of this system in nature (Keilin, 1925), and the poisonous effects of cyanide ion upon the system (Warburg, 1923; Dixon and Elliot, 1929, direct our attention to the possible modes of combination of cyanide ion with the constituents of the system. A compound of cyanide and hematin has been prepared by Ziemke and Miller (1901), who found its spectroscopic bands to be similar in position to those of cyanmethemoglobin. Anson and Mirsky (1928) describe two compounds of "reduced heme" with cyanide. A crystalline compound of cyanide and

methemoglobin containing one mole of hydrogen cyanide per iron equivalent of the pigment was prepared by von Zeynek (1901) who showed that the cyanmethemoglobin of Kobert (1891) is a methemoglobin derivative.

Balthazard and Philippe (1926) report an evanescent spectroscopic picture, seen during the course of a reduction of cyanmethemoglobin, which they ascribe to the intermediary formation of a compound of reduced hemoglobin with cyanide. They assign to cyanmethemoglobin the formula  $\text{HbO}(\text{CN})_2$ .

The electrometric study of hemoglobin initiated by Conant (1923) has opened another pathway for investigations of the reactions of certain blood pigments. From his studies, Conant deduced that methemoglobin differed from reduced hemoglobin only in that the iron of the former is in the deelectronated or ferric form. The iron of reduced hemoglobin is in the electronated or ferrous form. The iron in oxyhemoglobin and in carbon monoxide hemoglobin did not participate as an oxidant or reductant in this oxidation-reduction system.

As shown by Conant (1923) and Conant and Fieser (1924), methemoglobin and hemoglobin form a reversible oxidation-reduction system, the former being the oxidant and the latter the reductant. The oxidation-reduction potential of this system is well defined potentiometrically, varying logarithmically with the relative concentrations of methemoglobin to hemoglobin. The oxidation of the latter to the former, or removal of hemoglobin from the system in any way, as for example by its oxygenation or carbonylation, will raise the observed potential. The removal from the system of methemoglobin will lower the observed potential correspondingly.

Since we might expect the electrical behavior of either of these pigments when uncombined to differ from that exhibited as a cyanide derivative, the electrometric method was deemed suitable for the demonstration of combination between cyanion and the pyrrol-iron of either methemoglobin or hemoglobin.

#### *Preparation of Hemoglobin*

400 cc. of oxalated or defibrinated dog's blood was used. Illuminating gas was passed through the chilled sample for 15 minutes; 200 cc. was poured into each of two 250 cc. centrifuge bottles and after centrifuging without packing the corpuscles the serum was removed by means of a suction pipette. The corpuscles were

washed five times with  $\text{CO}_2$ -saturated, ice-cold 0.17 molar sodium chloride solution, filling the tubes each time to the original level. For the first four washings, the centrifugation was carried only to the point where the corpuscles occupied about one-half of the total volume. The last centrifugation was carried on until the corpuscles were packed and the supernatant fluid could be removed by decantation. The tubes were then inverted to drain for 10 minutes, after which they were filled to the original 200 cc. level with ice-cold,  $\text{CO}_2$ -saturated, distilled water and toluene added (as in the method of Heidelberger (1922)) to within such a distance of the top that, when a tight-fitting cork was inserted there was a large air bubble left to facilitate subsequent shaking. The packed mass of corpuscles was disintegrated throughout the fluid with a glass rod; the tubes were then stoppered and vigorously shaken for 5 minutes. Early in the shaking process a gel formed, but shaking was continued until the toluene was comminuted in the mass.

The tubes were left in the refrigerator for 12 hours, then transferred to the freezing room until the contents were frozen solid. They were then centrifuged, during which time the contents were allowed to thaw. Four layers were obtained:—toluene, toluene gel, saturated carbon monoxide hemoglobin solution, and crystals of carbon monoxide hemoglobin.

The toluene, toluene stroma combination, and saturated carbon monoxide hemoglobin solution were removed by a pipette with suction. The rim left by the toluenized stroma was thoroughly removed from the sides of the container by a plug of absorbent cotton on a wire.

The crystals were covered with  $\text{CO}_2$ -saturated ice water, toluene was added as before, the tubes stoppered, shaken, and immediately centrifuged. As a rule the toluene returned clear with little or no stroma material. The supernatant fluid was decanted and ice water was added, the tubes shaken, and placed in the refrigerator. The crystals settled to the bottom and the supernatant solution was barely tinged with hemoglobin.

For preparing the solutions, some of the crystals were transferred along with 50 cc. of distilled water to a tall aeration cylinder of about 500 cc. capacity fitted with a two-hole rubber stopper carrying a three-way stop-cock and a small separatory funnel. Suction was instituted through the three-way stop-cock, with periodic whirling of the cylinder to suspend the crystals. After ebullition by the dissolved and combined carbon monoxide had slackened, nitrogen (or hydrogen if electrometric pH was to be determined) was admitted through the stop-cock, the cylinder was shaken, and suction reinstituted. 3 hours of evacuation was found sufficient to completely reduce carbon monoxide hemoglobin, but the suction was left on for 3 hours longer until considerable diminution in the original volume of the solution had taken place. (Solid reduced hemoglobin has been prepared by suction evaporation of all the water present, and this solid when redissolved retains its power to combine reversibly with molecular oxygen as shown in Table I).

The reduced hemoglobin solutions prepared by this method were found to be

isoelectric (Table II) and to have an activity approximating 100 per cent (Table III).

TABLE I  
*Activity of Reduced Hemoglobin Dried in Vacuo at 100°C.*

Sample	Total (Hb)*	Active (Hb)†	Activity
	<i>mu/liter</i>	<i>mu/liter</i>	<i>per cent</i>
Dog	0.56	0.34	60.7
Human	1.98	0.66	33.3

\* Total hemoglobin was determined by the method of Barnard (1932b).

† Active hemoglobin was determined on the basis of its oxygen capacity.

TABLE II  
*pH of Reduced Hemoglobin Solutions from Carbon Monoxide Hemoglobin Crystals*

Before washing .....	6.121
After second washing .....	6.509
" fourth " .....	6.783

pH was determined by the hydrogen electrode in a Hastings (1921) electrometric titration vessel.

TABLE III  
*Activity of Reduced Dog Hemoglobin Solutions Prepared by Suction Evacuation of Carbon Monoxide Hemoglobin Crystal Suspensions*

Total (Hb)*	Total (Hb)†	Activity
<i>mu/liter</i>	<i>mu/liter</i>	<i>per cent</i>
0.70	0.71	101.4
2.75	2.71	101.5
0.26	0.26	100.0
1.87	1.53	81.8‡

\* Total Hb was determined by method of Barnard (1932b).

† Active Hb was determined from the CO capacity.

‡ This solution was prepared from crystals kept 49 days after preparation.

### *Experimental*

The effect of cyanide on the potentials of poised solutions of hemoglobin and methemoglobin was noted. Hydrogen cyanide was passed through solutions containing hemoglobin and methemoglobin (the latter formed directly in the solu-

tion by the addition of potassium ferricyanide) in an electrometric titration vessel (Hastings, 1921). In some experiments potassium cyanide was added to the solutions in place of hydrocyanic acid. Readings were made after constant potential had been reached, the chain in each case being:



The solutions were at all times kept under nitrogen, and in those instances where titrations were performed the reagents employed were evacuated and saturated with this gas previous to use.

TABLE IV

*The Effect of Hydrogen Cyanide and Carbon Monoxide on the Oxidation-Reduction Potential of the Hemoglobin-Methemoglobin System*

Preparation	(Hb)	(MetHb)	Gas	E.M.F. on saturated KCl-calomel scale	
				Point - Pt electrode	- Plate
	<i>mm/liter</i>	<i>mm/liter</i>			
1—dog	2.37	0.40	N <sub>2</sub>	—0.0931	
1— “			HCN	—0.2046	—0.2079
1— “			CO	Unpoised	Unpoised
2— “	3.0	3.0	N <sub>2</sub>	—0.0613	—0.0617
2— “			HCN	—0.1423	—0.1713
2— “			CO	Unpoised	Unpoised

Both solutions were buffered at pH 6.2 with 0.067 molar phosphate. The gases were passed through the solution in the order given.

### Results

The effects of passing hydrogen cyanide through solutions containing hemoglobin and methemoglobin on the electrode potentials developed by these solutions are shown in Table IV. Table V gives the results of measurement of the  $e_0$  value for monkey and dog hemoglobin as well as the effect of potassium cyanide on the potentials developed. In Fig. 1 is represented a titration of pig hemoglobin with ferricyanide in the presence and absence of cyanide. Similar results have been obtained with beef and with human hemoglobin (Barnard, 1933). Table VI gives the data on a control experiment in which the relative absence of effect of cyanide on the potentials of the ferricyanide-ferrocyanide system was demonstrated.



The addition of hydrogen or potassium cyanide to the methemoglobin-hemoglobin system caused a shift in the potentials evinced by this system to the negative side, indicating that the oxidant is removed

TABLE V

*The Effect of Alkali Cyanide on the Oxidation-Reduction Potentials of the Hemoglobin-Methemoglobin System*

Preparation	(Hb)	(MetHb)	(MetHbCN)	(CN)	$e_1$	$e_0$	$n$
3—dog	1.376	0.0	0.024	2000	-0.5912		
3— “	1.343	0.0	0.057	2000	-0.5895		
3— “	1.200	0.0	0.200	2000	-0.5762		
3— “	1.0	0.0	0.40	2000	-0.4806		
3— “	0.60	0.0	0.80	2000	-0.2601		
3— “	0.20	0.0	1.20	2000	-0.190?		
4— “	0.048	0.0	0.052	40	-0.3731		
5— “	0.080	0.040	0.0	0	-0.1869	-0.1690	1
5— “	0.040	0.080	0.0	0	-0.1516	-0.1695	1
1—monkey	1.520	0.480	0.0	0	-0.1686	-0.1491	2
1— “	1.160	0.840	0.0	0	-0.1532	-0.1492	2
1— “	0.920	1.080	0.0	0	-0.1443	-0.1484	1
1— “	0.560	1.440	0.0	0	-0.0946	(?)	(?)
1— “	1.736	0.0	0.264	1000	-0.4539		
1— “	1.496	0.0	0.504	1000	-0.410?		
1— “	0.560	0.0	1.440	1000	-0.240?		
1— “	0.152	0.0	1.848	1000	-0.2480		
6—dog	6.320	0.0	0.0	0	-0.2051		
6— “	5.670	0.650	0.0	0	-0.1623	-0.1295	1.7
6— “	5.150	1.170	0.0	0	-0.1556	-0.1295	1.5
6— “	4.670	1.650	0.0	0	-0.1457	-0.1295	1.7
6— “	3.870	2.450	0.0	0	-0.1315		
6— “	3.520	2.80	0.0	0	-0.1301		
6— “	2.820	3.50	0.0	0	-0.2833	(87.5 mm NaOH added)	

All concentrations expressed in millimols per liter and all potentials are in volts on the saturated KCl-calomel scale.

from participation in the system. When carbon monoxide was passed through the solutions of methemoglobin and hemoglobin which had been subjected to the action of cyanide, the potentials became

TABLE VI

*The Effect of Potassium Cyanide on the Oxidation-Reduction Potential of the Ferricyanide-Ferrocyanide System*

$(\text{K}_3\text{Fe}(\text{CN})_6)$	$(\text{K}_4\text{Fe}(\text{CN})_6)$	$(\text{CN})$	$e_1$
mm/liter	mm/liter	mm/liter	
10	10	0.0	-0.1729
10	10	66.7	-0.1708
10	10	100.0	-0.1688

unpoised. The hemoglobin present apparently retained its reductant position in the presence of cyanide. That hydrogen cyanide and so-

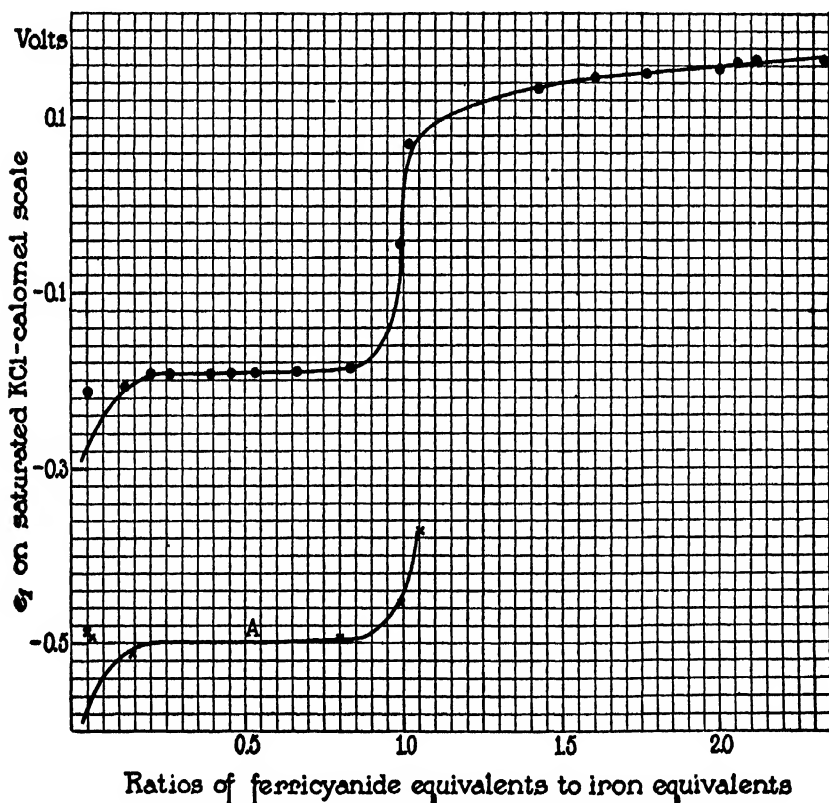


FIG. 1. The effect of cyanide on the oxidation-titration curve of pig hemoglobin. Ordinates give  $e_1$  in volts on the saturated calomel scale. Abscissae give the ratios of molarity of ferricyanide added to molarity of hemoglobin. A represents the titration curve in the presence of 100 millimolar sodium cyanide.

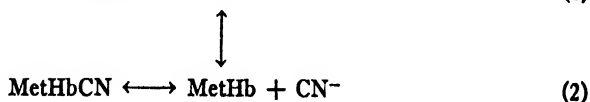
dium cyanide did not prevent the formation or dissociation of carbon monoxide hemoglobin was demonstrated spectroscopically and by measurement of the carbon monoxide capacity of hemoglobin solutions containing cyanide (Table VII). It is apparent from these results that the effects of cyanide on the potentials of the methemoglobin-hemoglobin system can best be attributed to its combination with methemoglobin, and that if cyanide does combine with hemoglobin, it is not with that portion of the molecule which gives the latter its spectroscopic picture, its capability of combination with gases, and its reductant activity. In those instances where cyanmethemo-

TABLE VII

*The Effect of Cyanide on the Carbon Monoxide Capacity of Hemoglobin Solutions*

Sample	$p_{CO}$	(CN)	$t$
		<i>mm/liter (NaCN)</i>	
Sheep	116.6	0.0	20.2
"	117.5	40.0	20.2
Human 1	274.4	0.0	21.0
" 1	243.7	200.0	20.4
" 2	186.6	0.0	
" 2	169.0	5000.0	
Dog	126.1	0.0	20.6
"	121.9	333.3(HCN)	20.4

globin results from the treatment of oxyhemoglobin solutions with cyanide, as after long standing or after oxyhemoglobin has been heated with cyanide, it is probable that methemoglobin is formed as an intermediary in the conversion represented by the equations:



That the formula of cyanmethemoglobin in solution corresponds to the crystalline compound prepared by von Zeynek (1901) and that there is a ratio of one equivalent of cyanide to one iron equivalent of methemoglobin is deducible from the following experiment:

10 cc. of a solution containing 13.2 millimolar pig methemoglobin and 2.04 millimolar pig hemoglobin was titrated electrometrically with 77 millimolar potassium cyanide solution. The results of this titration are represented in Fig. 2. The potentials recorded in that vicinity of the curve where the ratio of cyanide to methemoglobin iron was unity were unstable, but above and below this point they were suffi-

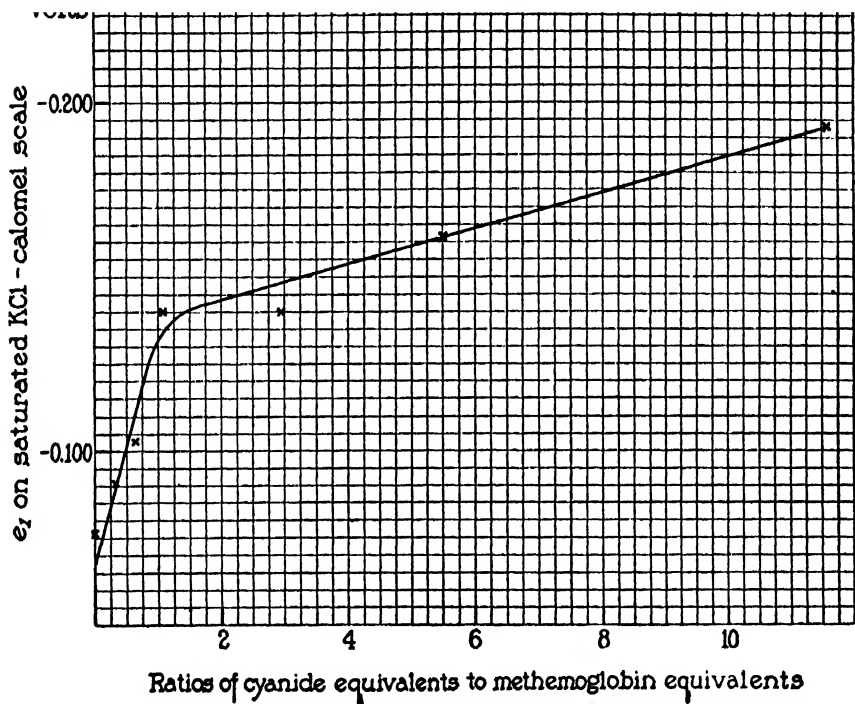
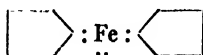


FIG. 2. The effect of cyanide on the oxidation-reduction potential of the pig methemoglobin-hemoglobin system. Ordinates give  $e_1$  in volts on the saturated calomel scale. Abscissae represent the ratio of the molarity of cyanide to that of methemoglobin.

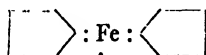
ciently constant to allow for an interpolation to the point where an inflection occurs in the curve. This inflection is presumably the end-point of reaction (2), and the fact that it takes place before two equivalents of cyanide are added indicates that there are not two equivalents of cyanide in cyanmethemoglobin as deduced by Baltazard and Philippe (1926).

That compound formation between cyanide and the iron of the pros-

thetic nucleus of hemoglobin would be improbable is in harmony with the nature of the electronic formula previously assigned to this compound, (Barnard, 1932a)



which from its depicted structure, would have no free valency electron with which to form ionic combinations.<sup>1</sup> By deelectronation of the hemoglobin iron, however, an electropositive radicle, methemoglobin, results. It is probable that this radicle.



might form electronic linkages with cyanion or with other electro-negative ions.

#### *The Effect of pH on the Oxidation-Reduction Potential of the Methemoglobin-Hemoglobin System*

In order to control the experiments in which the effect of alkali cyanide was studied, the effect of alkali hydroxide on the electrical behavior of the methemoglobin-hemoglobin system has been determined.

Conant and Fieser (1924) found that the  $e_0$  value for this system became more negative with increasing pH. The opinion advanced by them that methemoglobin is a weaker acid than hemoglobin, is not consistent with the data presented by Hastings (1928) which show that between pH 7.0 and 7.8 no difference in the base-combining powers of the two pigments is detectable.

#### *Experimental*

Isoelectric carbon monoxide hemoglobin was brought into solution as reduced hemoglobin by the removal of carbon monoxide by evacuation. The concentration of the reduced pigment was determined from its carbon monoxide capacity by the method of Van Slyke and Neill (1924). The solution was transferred to the electrometric titration vessel under nitrogen and the desired quantity of meth-

<sup>1</sup> The iron is represented with only six electrons to convey the idea of its ferrous state, although it is probable that a shared electron from each of the pyrroles would bring the outer shell to eight.

moglobin formed in it by the addition of an equivalent of potassium ferricyanide. Sodium hydroxide solution, which had been evacuated and saturated with nitrogen was added to the solution in the titration vessel from a micro buret, and after

TABLE VIII

*The Effect of pH on the Oxidation-Reduction Potential of the Methemoglobin-Hemoglobin System*

mm NaOH	BHb/Hb	pH	$\epsilon_1$	$\frac{\Delta \epsilon_o}{\Delta pH}$
Experiment 5				
(Hb) = 3.52 mm/liter (MetHb) = 2.80 mm/liter				
00.0	0.0	6.81	-0.1301	0.088
22.3	0.352	7.07	-0.1531	(0.361)*
38.0	0.600	7.17	-0.1891	0.080
58.7	0.929	7.34	-0.2027	0.053
100.0	1.580	7.67	-0.2201	(0.103)*
150.1	2.360	8.05	-0.2594	0.041
200.0	3.160	8.42	-0.2756	0.050
300.0	4.740	9.25	-0.3121	0.062
400.0	6.330	10.50	-0.3404	
Experiment 6				
(Hb) = 1.15 mm/liter (MetHb) = 1.604 mm/liter				
00.0	0.0	6.81	-0.1010	0.063
10.0	0.364	7.08	-0.1180	(0.109)*
20.7	0.750	7.24	-0.1354	0.060
35.1	1.270	7.50	-0.1509	0.069
52.5	1.920	7.84	-0.1775	0.065
72.0	2.600	8.20	-0.2009	0.071
94.7	3.450	8.60	-0.2294	(0.025)*
99.8	3.600	8.70	-0.2319	0.087
130.0	4.700	9.24	-0.2381	0.069
179.0	6.500	10.05	-0.2469	
200.0	7.270	10.25	-0.2626	
250.0	9.090	10.51	-0.2767	
300.0	10.900	10.62	-0.3021	

\* Discarded from average.

each increment the potential was recorded with the same chain as described above. The pH of the solutions was calculated from data relating it to the base bound by reduced dog hemoglobin at different ionic strength (unpublished data). An identity in the base-combining powers of dog methemoglobin and dog hemoglobin has been assumed.

### Results

As found by Conant and Fieser (1924), the potentials of the system became more electronegative as the alkalinity increased. Experiment 5 (Table VIII) in which the solution titrated contained an excess of hemoglobin over methemoglobin gave 0.062 as a calculated value

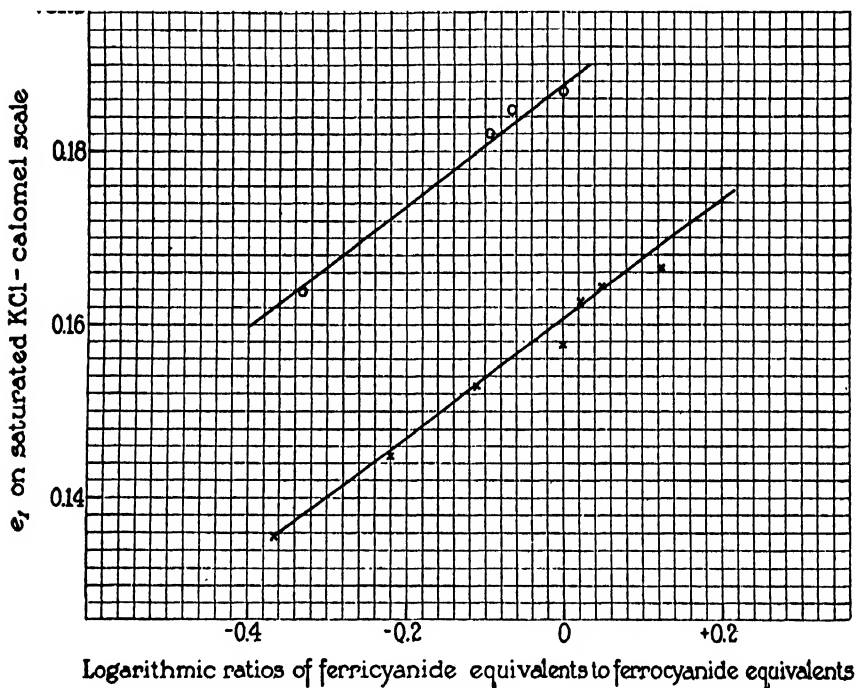


FIG. 3. The  $n$  slope for the ferricyanide-ferrocyanide oxidation-reduction system. Ordinates give  $e_1$  in volts on the saturated calomel scale. Abscissae give the logarithmic ratios of the concentration of ferricyanide to ferrocyanide. The upper line gives the position for the slope of this system in solutions containing allantoin, the lower line in solutions of methemoglobin as calculated from Experiment 3.

for  $\frac{\Delta e_o}{\Delta \text{pH}}$  between pH 6.81 and 9.25. In Experiment 6 (Table VIII) an excess of methemoglobin existed and the value obtained between pH 6.81 and 9.24 was 0.069.

An attempt to recalculate the values of  $\frac{\Delta e_o}{\Delta \text{pH}}$  for this system from the work of Conant and Fieser by taking into account the buffer values of

hemoglobin in correcting their pH values has led to results in good agreement with those found in this study.

Peculiarly enough, in the titration of the rather concentrated pig hemoglobin solution (15 millimolar) (Experiment 3) the value of  $n$  for the methemoglobin-hemoglobin system was 4 and not as in all other experiments in the neighborhood of 1 or at the most 2. That the calculated value in this pig hemoglobin titration is not due to an inaccuracy of the data we can assume from a consideration of Fig. 3, where the  $n$  value of the ferricyanide-ferrocyanide system in this same experiment is 1 and compares excellently with the slope calculated from other data utilizing this system (Barnard, 1931). We do not, at this time, consider the value of  $n$  in this particular oxidation-reduction titration as fortifying Adair's (1925) hypothesis as to the oxygenation of hemoglobin any more than in 1928, when we first measured and secured the value of  $n = 1$  in the electrochemical equation was it considered an objection to the theory of Hill (1910). Conant and McGrew (1929), from whose paper it is inferred that the electrometric measurements of  $n$  in the oxidation-reduction equation

$$e_o = e_1 - \frac{RT}{nF} \log_e \frac{(\text{MetHb})}{(\text{Hb})}$$

are comparable to those of  $n$  in Hill's equation

$$\frac{(\text{Hb})_n \times (\text{O}_2)^n}{(\text{HbO}_2)_n} = \frac{1}{K'}$$

assigned to  $n$  in the former case a value of 2 as determined by Conant and Scott (1928). Conant and Pappenheimer (1932) have redetermined the value of  $n$  in the oxidation-reduction equation and find that it may be in the vicinity of 1.

A consideration of Fig. 3 will also show that although the slopes for the ferricyanide-ferrocyanide system in the two differing experiments are comparable, the  $e_o$  value in the presence of the concentration of methemoglobin extant in Experiment 3 is 125 mv. lower than the characteristic value for this system. This is explained on a basis of the enormous ionic strength effects of hemoglobin solutions, which, as will be shown in a subsequent report, appear to behave in their salt effects as strong polyvalent electrolytes.

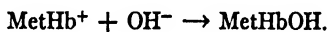


## DISCUSSION

Since the addition of alkali causes a change in the oxidation-reduction potential of solutions containing methemoglobin and hemoglobin it is obvious that one or both of these substances is in some way altered by the addition. Is this alteration in the globin fraction? Two facts would indicate that it is not. First, there is a comparable slope for the hematin-reduced hematin system where no globin is present. Second, the identity of base-binding powers of methemoglobin and hemoglobin would point against the existence of any difference in their respective globins. The methemoglobin molecule does, however, change its spectroscopic picture on treatment with alkali, whereas reduced hemoglobin does not change in this respect within the pH range studied. It is therefore tempting to ascribe the direction of the slope to that constituent of the system which gives other evidence of lability. Though it is recognized that other possibilities may exist, the direction and magnitude of the slope can best be explained by the formation on the part of methemoglobin of an electropositive ion,



which in the presence of hydroxyl, just as in the presence of cyanion, forms a poorly dissociated compound, in the manner:

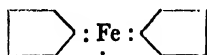


It is evident that the methemoglobin ion might be capable of combination with many electronegative radicles. Such a combination between methemoglobin and nitrite is undergoing study at present.

*The Interrelationship of the Hemoglobin Derivatives*

The electronic formula (A)

(A)



was suggested for the prosthetic group of methemoglobin and since the prosthetic group of the latter is probably identical with hematin (Kuster, 1910), the formula (A) will be used for the latter compound as well. (A) is converted by cyanide to a compound (B), the prosthetic group of which may be represented as

(B)

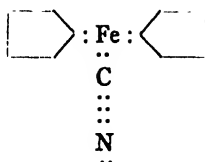


TABLE IX

*The Spectrophotometric Comparison of Cyanhematin and Cyanmethemoglobin  
Percentage Transmission*

$\lambda$	Preparation 1		Preparation 2		Preparation 3	
	HmCN	MetHbCN	HmCN	MetHbCN	HmCN	MetHbCN
460	2.97	2.85	5.00	7.23	10.75	7.90
480	9.75	9.66	15.50	16.65	20.66	17.04
490						28.75
500	14.18	13.10	30.00	30.08	27.60	29.75
510				29.10		31.25
520	14.20	12.68	29.25		22.70	25.50
530					20.17	19.88
540	9.95	8.55	26.00	24.13	18.50	17.75
550				19.75	22.40	19.22
560	12.33	11.05	22.80	18.19	24.40	24.0
580	23.93	20.44	28.25	22.70		37.33
590					49.51	49.00
600	41.68	36.15	36.00	35.03		
620	56.13	54.50	53.75	53.75		
640	67.13	62.50	68.00	74.13		
660	70.00	67.75	79.50	88.50		
680	71.25	69.50	87.50	93.00		
700	74.00	70.25	88.50	95.00		
720	68.50	72.25	88.00	93.00		

Preparation 1. (HmCN) = 0.064 mM/liter, prepared by treating oxyhemoglobin with 0.1 M HCl, neutralizing with KCN and diluting to the concentration given. (MetHbCN) = 0.066 mM/liter, prepared by dissolving 0.1102 gm. of dry methemoglobin in 1 liter of 1 M KCN solution.

Preparation 2. The concentrations of cyanhematin and cyanmethemoglobin were not determined but were identical since they were prepared from the same solution of oxyhemoglobin.

Preparation 3. The concentrations of cyanhematin and cyanmethemoglobin were identical but in the case of cyanhematin, the globin was digested with pepsin-hydrochloric acid mixture to preclude cyanmethemoglobin resynthesis, although there is probability of combination between the hematin and some of the digestion products of the globin.

There should be some similarity between cyanmethemoglobin and cyanhematin since they would both contain the same prosthetic group. Table IX gives the data obtained in a spectrophotometric study of these compounds. The percentage transmission at given wave lengths is similar for both substances. This confirms the spectroscopic findings of Ziemke and Miller (1901).

That the prosthetic nucleus of cyanhematin is a ferric cyanide is inferred from the following observations:

(a) Passing  $\text{CO}_2$  through cyanhematin solutions precipitates hematin.

(b) Treatment of cyanhematin solutions with barium hydroxide precipitates a powder which retains the orange color of the original solution. Since the barium linkage is undoubtedly on the carboxyls of the pyrrol groups, the point of cyanide attachment is probably on the iron. In the case of cyanmethemoglobin, it was found that the latter was not precipitated from solution by barium hydroxide and this is readily explicable on the basis of the fact that the pyrrol carboxyls are already occupied by globin.

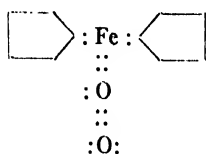
It is assumed that, in basic solution, (A) forms alkaline derivatives. In the case of methemoglobin it was assumed that this derivative was a ferric hydroxide. Certain points of similarity in alkaline methemoglobin and alkaline hematin furnish evidence for the applicability of the same formula to the latter substance as well. Among these points may be mentioned the indicator nature of both pigments and the coincidence of the  $\frac{\Delta e_o}{\Delta \text{pH}}$  slope for the systems in which each participates as oxidant. The prosthetic nucleus of alkaline methemoglobin and alkaline hematin will therefore be represented as



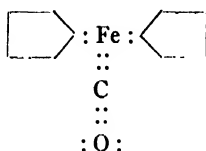
Since hematin and methemoglobin may be electronated to form reduced hematin and hemoglobin respectively, the electronated form of (A) will be represented as (D). From an analysis of the structure of the nucleus



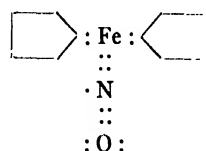
we could predict its electronegativity, and the formation by it of polar valent compounds with electropositive radicles. This behavior is realized in the hemochromogens, which combine with carbon monoxide and nitric oxide and in hemoglobin which combines with molecular oxygen as well. Such compounds as (D) with oxygen-containing radicles have been presented (Barnard, 1932a) as:



Oxyhemoglobin



Carbon monoxide hemoglobin



Nitric oxide hemoglobin

Configurations such as the above cannot be drawn for the problematic compounds ethylene hemoglobin and nitrous oxide hemoglobin, and evidence against the existence of these compounds was secured in a previous investigation (Barnard and Hastings, 1930).

#### SUMMARY

The potentiometric method was applied to the study of the influence of cyanide and of hydroxyl ion on methemoglobin. Both of these ions appear to combine with the iron of the methemoglobin molecule and reduce its oxidant activity. From the magnitude of the effect produced by cyanide and by variation in pH on the oxidation-reduction potential of the methemoglobin-hemoglobin system, it is concluded that cyanmethemoglobin and alkaline methemoglobin are undisassociated ferric compounds, the first with cyanide and the second with hydroxyl.

Electronic formulas, based on the electrical properties of the hemoglobin derivatives, are suggested.

The author wishes to express his gratitude to Dr. A. Baird Hastings and Dr. A. J. Carlson, in whose laboratories the major portion of the experimental work was done.

#### *Explanation of Symbols*

Hb            = hemoglobin  
HbO<sub>2</sub>        = oxyhemoglobin

MetHb = methemoglobin

MetHbCN = cyanmethemoglobin

HmCN = cyanhematin



= pyrrol ring

$e_0$  = the potential characteristic of an oxidation-reduction system

$e_1$  = the observed potential

$n$  = the number of electrons involved in the transformation of oxidant to reductant or the degree of aggregation of hemoglobin in Hill's (1910) equation

( ) = denotes concentration

$F$  = the Faraday

$R$  and  $T$  = the gas constants

$K$  = Hill's (1910) constant ( $4 \times 10^{-4}$  at pH = 7.3)

$\lambda$  = wave length

all concentrations are expressed as millimols per liter unless otherwise stated. The mol of any hemoglobin derivative is taken as equal to one iron equivalent of the derivative.

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# STIMULATION BY THE MINERAL ACIDS, HYDROCHLORIC, SULFURIC, AND NITRIC, IN THE SUNFISH EUPOMOTIS

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## I

In previous studies on chemical stimulation of aquatic animals it has been assumed that the response measured is a function of the intensity of stimulation of the receptor surface, and that the response may be correlated with the nature of the stimulating substance which initiates a series of energy changes in the heterogeneous system consisting of the chemical environment of the animal and the receptors concerned. The last event in the series activates the neuromuscular mechanism, the efficiency of which is assumed constant in any individual under ordinary conditions. Adaptation and injury must be absent and the energy changes at the receptor surface must be reversible, since the responses are easily reproducible.

The original energy change is initiated by the alteration of the ionic or molecular equilibrium and strength of the animal's chemical environment. Stimulation by the primary aliphatic alcohols is evidently a direct effect upon the receptor surface brought about by the addition of alcohol molecules to the environment and by their specific orientation in relation to that surface (Cole and Allison, 1930-31). Since the polar hydroxyl group ( $-OH$ ) in the alcohols appears to be constant, the predominate factor in stimulation is the field of force around the  $R$  group. Similarly, stimulation by the normal primary aliphatic acids in the sunfish (Allison, 1931-32) may be best correlated with the non-polar portion of the molecule, except for the first member of the series where it is necessary to consider the higher potential of the

\* A part of the expenses of this investigation was met by a grant from the National Research Council for 1932.



polar group as playing a significant rôle. Again, stimulation by the salts of the normal primary aliphatic acids in the barnacle appears to be chiefly correlated with the non-polar portion of the molecule (Cole, 1931-32). On the other hand, stimulation by the same acids in the fresh-water catfish *Schilbeodes* is dependent upon the potential of the polar group, or upon the ability of that group to change the ( $H^+$ ) of the environment.<sup>1</sup> Such a dependence might be predicted because of the great susceptibility of the catfish to slight changes in ( $H^+$ ) (Cole and Allison, 1931-32). As the length of the carbon chain increases, the non-polar portion of the molecule may begin to play a rôle.

In any complete series of polar organic molecules it is likely that stimulation may be traced ultimately to the activity of the polar group, or to the field of force around the non-polar group, or to both. One or the other factor may predominate, or each may play a significant rôle. It is logical therefore to consider separately the field of force around the radical  $R$  and the potential of the polar group such as  $-OH$ ,  $-COOH$ ,  $-CHO$ ,  $-NH_2$ , etc. If the stimulating substance is dissociated into ions which in themselves may be considered entirely polar, such as are produced from inorganic compounds, it would also be logical to consider separately the fields of force around each ion.

## II

Further experiments have been done on the sunfish *Eupomotis gibbosus*, to determine whether disturbances in the chemical environment initiated by the anions of the strong inorganic acids, hydrochloric, sulfuric, and nitric, would alter the relationship between ( $H^+$ ) and reaction time as demonstrated for hydrochloric acid alone (Allison, 1931-32); the procedure used was the same as that already described. Features of the method are the constant rate of flow of solution and spring water over the fish, allowance of ample time for recovery of the fish between trials, thereby preventing any adaptation, and measurement of the ( $H^+$ ) of each solution by the quinhydrone electrode. Two series of animals were used over a period of 6 months.

<sup>1</sup> Unpublished results obtained in 1930.

In one series two observations were made on each of eight fish for each concentration ( $n = 16$ ), and in the other series two observations on each of ten fish ( $n = 20$ ), resulting in 628 observations on eighteen fish. The data for hydrochloric acid where  $n = 16$  are taken from Allison (1931-32). Freshly collected fish responded more quickly than those well adapted to laboratory conditions. Relatively, however, the relation between rate of response and concentration was the same for each group. By adding 1.25 seconds to the reaction times of freshly collected fish the two sets of data become coincident. As before, the actual reaction times have been corrected by subtracting 5.0 seconds, which is considered to be the theoretical shortest possible reaction time obtainable from any individual. Confirmation of this value is found by extrapolating the ( $H^+$ ) where  $y = 0$ , corresponding to a pH of 1.88 (see Fig. 2). In the preliminary tests it was regularly noted that a pH of 2.0 or less was injurious to the fish and caused an increase in reaction time, indicating that other processes were occurring besides stimulation.

### III

When an acid, such as hydrochloric, is added to the chemical environment of the fish, not only is the ( $H^+$ ) changed but also the ( $Cl^-$ ), and a new equilibrium is established throughout the whole system. The degree of shift in the ionic and molecular equilibrium of the system may be related to the effective concentration of one of the ions or the other. If the animal's response is a function of the ( $H^+$ ) alone, then no difference would be observed between the stimulating efficiency of different acids used to alter the concentration of that ion. If, however, the specific nature of the anion plays the predominate rôle, then each of the acids would give different results. For the sunfish the data secured show clearly that stimulation by hydrochloric, sulfuric, and nitric acids is primarily dependent upon the ( $H^+$ ) within the range tested (Table I). In Fig. 1 are plotted thirty-four average reaction times as related to the ( $H^+$ ) produced in the environment by adding hydrochloric, sulfuric, and nitric acids. The smooth curve is drawn through coordinates calculated from the equation of the line in Fig. 2. That line was laid off through the middle of a parallel band of points with a

TABLE I

*Corrected Reaction Times of Sunfish to Different Hydrogen Ion Concentrations in Solutions of Hydrochloric, Sulfuric, and Nitric Acids Made in Spring Water*

(H <sup>+</sup> ) conc. $\times 10^4$	RT-5	P.E. RT-5
Hydrochloric		
	sec.	
6.31*	5.60	0.429
10.00	4.75	0.296
10.72*	4.36	0.227
13.49*	3.46	0.127
16.22	4.31	0.344
21.88	3.57	0.218
25.71	3.43	0.240
31.63*	2.48	0.143
33.12*	2.42	0.184
35.49	2.50	0.276
Sulfuric		
7.08	5.88	0.575
9.55*	4.72	0.351
12.89	4.63	0.286
13.81*	4.24†	0.226
20.90	3.21	0.269
22.91*	3.37†	0.149
24.55*	2.96	0.196
30.91*	2.18	0.129
31.63	2.21	0.166
Nitric		
5.62	5.73	0.362
6.31	5.51†	0.285
6.46	5.18†	0.246
6.60	5.57	0.416
10.48	4.50	0.241
10.97*	4.83†	0.207
16.22	4.27†	0.190
16.99	3.95	0.235
18.20*	4.07†	0.198
21.88	3.76	0.213
21.88	3.52	0.210
23.99*	3.20†	0.152
25.12	3.26†	0.175
26.31	2.13	0.137
37.16	2.52	0.166

$n = 20$  except in cases indicated by \*, where  $n = 16$ . The reaction times for freshly collected, non-adapted fish, indicated by †, have been increased by 1.25 sec. to make them coincident with the other data.

uniform distribution on each side. There is evidently no specific effect of the anions on the reaction time. It is often assumed, and

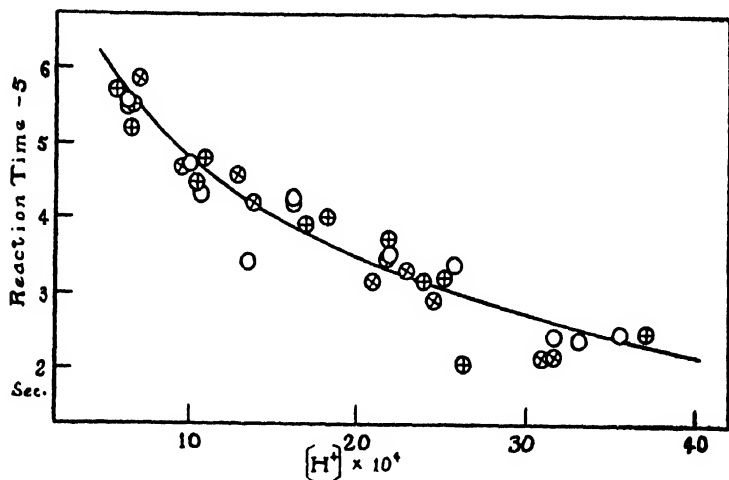


FIG. 1. The exponential relationship between corrected reaction times and  $(H^+)$ , as shown by the sunfish when stimulated by various concentrations of hydrochloric ( $\circ$ ), sulfuric ( $\otimes$ ), and nitric ( $\oplus$ ) acids. The smooth curve is drawn through coordinates calculated from the equation of the line in Fig. 2.

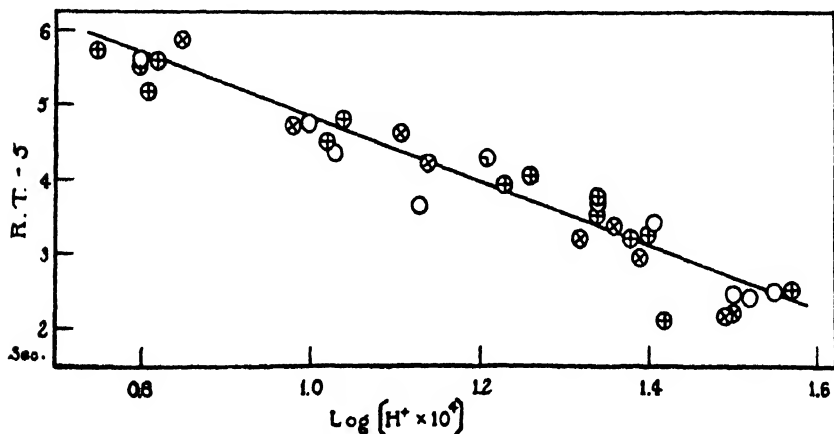


FIG. 2. Logarithmic plot of the same data as in Fig. 1. The linear relationship may be expressed by the equation:  $RT - 5 = -4.3 \log (H^+ \times 10^4) + 9.118$ . (The symbol  $\oplus$ ) represents coincident points for hydrochloric and nitric acids.)

probably correctly, that the hydrogen ion exerts a more or less direct effect upon the living system by shifting the  $(H^+)$  one way or

the other from the isoelectric point of some constituent in that system. Whether or not such an effect is present in the sunfish cannot yet be proved, but it is interesting to note that as the  $(H^+)$  increases geometrically the reaction time increases arithmetically. In other words,

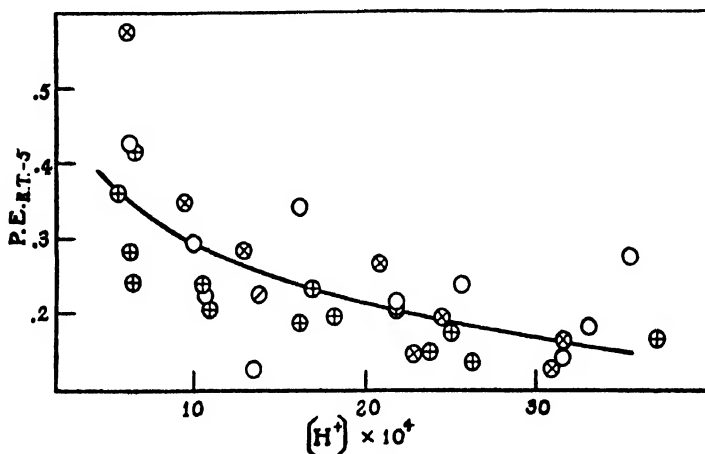


FIG. 3. The exponential relationship between the probable errors of the corrected reaction times and  $(H^+)$ . Same symbols as in Fig. 1. The smooth curve is drawn through coordinates calculated from the equations of the lines in Figs. 2 and 4.

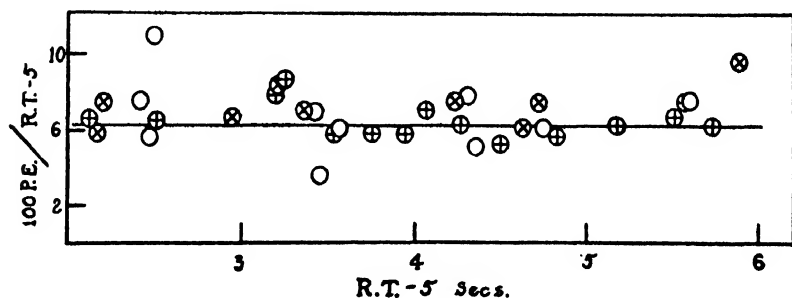


FIG. 4. The linear relationship between the percentage probable errors of the corrected reaction times and the corrected reaction times. Symbols as in Fig. 1.  $y = 6.2$ .

the reaction time is a logarithmic function of the  $(H^+)$ . Any disturbance of the equilibrium between receptor and the environment produced by the chloride, sulfate, and nitrate ions must be itself measured by the  $(H^+)$  of the solution.

Variations in the response as shown in Figs. 1 and 2 are assumed to be distributed uniformly about the theoretical lines. It is known that those variations are not due entirely to observational error or to experimental technique. They must be primarily intrinsic variations of the mechanism that determines the animal's response to the stimulus. If the efficiency of that mechanism does not change over the range of  $(H^+)$  tested, then the percentage variation of the mean reaction times should be constant when the number of observations and the number of animals are constant. That this is true is shown in Fig. 4. Percentage variation in reaction time is independent of the  $(H^+)$  over the range tested. To show that an exponential relationship also exists between  $(H^+)$  and the latitude of variation a smooth curve was drawn in Fig. 3 through coordinates calculated from the equations of the lines in Figs. 2 and 4. The general trend of the actual probable errors of the reaction times is thus indicated. Again no differences between the effects of hydrochloric, sulfuric, and nitric acids are noticeable.

In connection with the variations in response above noted, it should be emphasized that the data presented were obtained at different times over a period of 6 months, and on individuals obtained at different times from three different localities. Some of the fish were well adapted to laboratory conditions, but a few were used immediately after collection. No difference however in the exponential relationship between  $(H^+)$  and reaction time was apparent. It was only necessary to recognize the lower threshold for the freshly collected fish, and to make the proper correction in the reaction times. The percentage variation in response was the same for both groups.

#### SUMMARY

1. The stimulating efficiency of hydrochloric, sulfuric, and nitric acids has been measured in the sunfish *Eupomotis gibbosus*, by a method which reduces experimental errors to a minimum.

2. The results show that stimulation by these acids is primarily dependent upon the  $(H^+)$  produced in the animal's aquatic environment, and that the reaction time is a logarithmic function of the  $(H^+)$  within the range tested expressed by the equation:  $(RT-5) = -4.3 \log (H^+ \times 10^4) + 9.118$ .

3. Any effect of the chloride, sulfate, and nitrate ions must itself be measured by the ( $H^+$ ).

4. Variation in the reaction time is also a logarithmic function of the ( $H^+$ ), and the percentage variation is independent of the ( $H^+$ ) over the range tested.

5. Freshly collected fish show a lower threshold for stimulation as determined by the ( $H^+$ ) than do fish adapted to laboratory conditions, but relatively the reaction times of the two groups are the same.

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# SPECIFIC NERVE IMPULSES FROM GUSTATORY AND TACTILE RECEPTORS IN CATFISH

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Investigations in recent years have indicated a certain degree of quantitative specificity of impulses in afferent nerve fibers related to structural properties of the fibers (Erlanger, Bishop, and Gasser, 1926; Erlanger, 1927; Erlanger and Gasser, 1930). This specificity of impulses appears to be correlated, in part, with specific sense modalities (Matthews, 1929; Adrian, 1930, 1931; Adrian, Cattell, and Hoagland, 1931; Hoagland, 1932).

In a recent investigation, for example (Hoagland, 1932), action potentials from single nerve fibers supplying individual receptors in the skin of the frog were examined. Of forty-five receptive endings, thirty-two were found to respond only to light touch and not at all to 20 per cent acetic acid or to crushing, while thirteen responded only to these latter stimuli but not to light touch. The "touch" endings were not destroyed by the acid since they responded to light contact, giving characteristic action potentials in their supplying fibers during the time that the acid was on the skin. Ten other endings responded to both forms of stimulation. The action potentials initiated by stimulating the touch receptors as recorded by a Matthew's amplifier and oscillograph in conjunction with a camera, a loud speaker, and a standing wave screen, were typically of large amplitude and high speed in 75 per cent of the cases,—the *A*-type impulses of Erlanger and Gasser. Impulses initiated by stimuli which in man would be called painful travelled at a slower speed and were only from 5 to 50 per cent of the amplitude of those from fibers supplying tactile organs—the *B*- or *C*-type impulses of Erlanger and Gasser. In about a quarter of the cases this classification did not seem to hold. It was suggested that the specificity, probably correlated with diameter of



nerve fiber, might be a basis for epicritic and protopathic sensibility in the frog.

W. J. Crozier recently pointed out to me that catfish are known to be very sensitive to chemical stimulation and that an investigation of impulses set up by stimulating taste-buds located in the barbels might be of interest. The barbels of *Ameiurus*, as well as the lips, mouth, and skin of the flank possess numerous typical taste-buds (Herrick, 1901, 1903; Olmsted, 1920; May, 1925) and experiments involving responses to gustatory stimuli indicate clearly that these organs are extremely sensitive to sapid substances in the water (Herrick, 1903; Parker and Van Heusen, 1917).

### *Responses to Mechanical Stimulation*

The facial nerve complex of *Ameiurus nebulosus* Les., described in detail by Herrick in his paper of 1901, was bared by removing the eye in freshly decapitated catfish. The nerve leaves the skull sub-orbitally and sends a number of branches forward to the upper and lower lips and to the barbels. By clearing away connective tissue the myelinated branches of the nerve, some 5 to 8 mm. in length, may be separated from each other and their electrical responses tested by stimulating the skin supplied by them. Facial nerves in some twenty catfish were examined by drawing branches of the nerves across silver, silver-chloride electrodes connected through two amplifiers containing in all eight valves, resistance-capacity coupled, to a Matthews' iron armature oscillograph (Matthews, 1928). The impulses were recorded by means of a standing wave screen, a loud speaker connected to the output amplifier through an auxilliary amplifier, and a camera using moving bromide paper.

After drawing a branch of the nerve to be tested across the electrodes, a map of the cutaneous area of sensitivity supplied by it was determined by touching the barbels and lips and listening to the bursts of impulses on the loud speaker. In this way the sensitive areas for each branch of the nerve were clearly determined. The nerve and incision were kept bathed with Ringer's solution and the lips and barbels were stimulated while immersed in water. They were found to be extremely sensitive to mechanical stimuli—bursts of rapid impulses of large amplitude being set up in response to slight disturbances in

the water. In time relations the impulses were typical of the *A*-type impulses of Erlanger and Gasser (1930) produced in the cutaneous nerves of frogs in response to mechanical stimulation of the skin (Adrian, 1931; Hoagland, 1932).

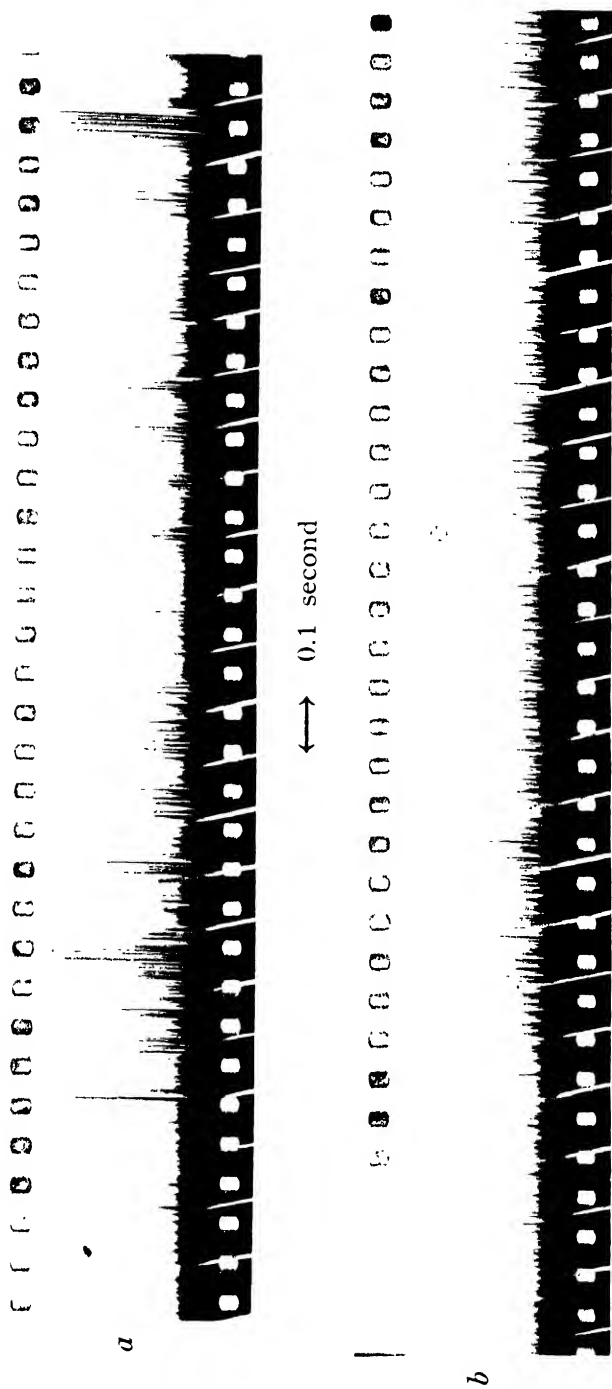
Fig. 1 shows typical records of action currents in response to (*a*) touching the barbels, (*b*) to the impact of a gentle stream of water from a pipette discharged beneath the water, a centimeter from the preparation towards a barbel at a rate of 5–10 cm. per second, and (*c*) to ripples in the water. In some preparations reflex bendings of the barbels occurred spontaneously. These movements were accompanied by bursts of impulses, an example of which is shown in Fig. 1 *d*. It is impossible to say whether impulses such as those of 1 *d* were produced exclusively from pressure receptors stimulated by the movement of the barbel through the water or by muscle spindles in the barbel. Both types of receptors may have been involved. Owing to the sensitivity of the barbel to relative motion of the water it seems certain that tactile receptors were stimulated.

No responses were elicited from the cutaneous receptors of the barbels or lips by tuning forks pressed against the outside of the dish or thrust into the water bathing the preparation. Forks of 100, 200, and 250 double vibrations were tried.

### *Rheotropism*

Groups of *Ameiurus* (at least two fishes), when in an aquarium with which they were familiar, were found to orient and swim treadmill fashion against a stream of water entering at one end of the tank and passing out at the other. This phenomenon is manifest when care is taken to eliminate influences of light since *Ameiurus* appears to be negatively phototropic. Maps of the cutaneous areas supplied by the facial nerves on the two sides of the head showed complete bilateral symmetry for the distribution of sensitivity. The forward parts of the large maxillary barbels especially at the middle third of their length were found to be extremely sensitive to mechanical stimuli, as judged from the volume of impulses initiated by currents of water and by stroking the skin.

This symmetry of sensitivity furnishes a basis for rheotropic orientation according to Loeb's well known mechanism of bilateral equaliza-



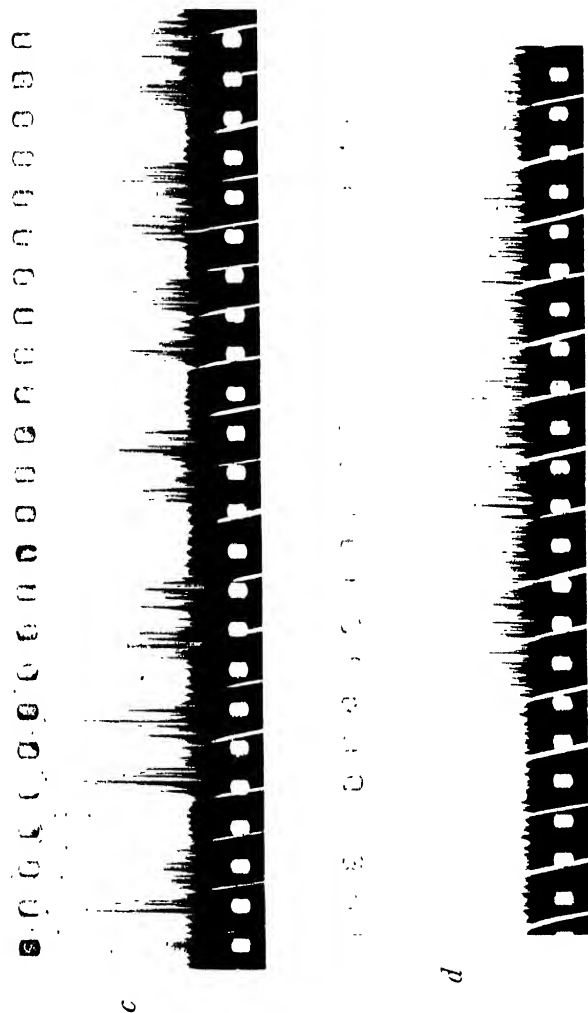


FIG. 1. Typical impulses from an immersed barbel of *Amiaurus* in response to mechanical stimulation. The lips and barbels all give similar responses and are about equally sensitive.

- (a) Bursts initiated by touching the skin with a feather. The response is the same for immersed barbels as for those in the air.
- (b) Effect of a current of water (5-10 cm., second) from a pipette. A similar effect is produced by pressing against a barbel.
- (c) Bursts set up by rippling the water.
- (d) Impulses due to reflex movement of the barbel in the water.

tion of central excitatory states. The findings are consistent with the experiments of Jordan (1917) on the rheotropism of the grouper fish *Epinephelus*, which he found to depend upon integumentary sensitivity, especially the sensitivity of the lips.<sup>1</sup> Maxwell (1921 *a*, 1921 *b*) has shown that contact stimuli applied to the skin of the snout and head produce systematic orienting movements of the swimming appendages of the skate and dogfish. The effects are also produced by streams of water from a pipette. The importance of rheotropic orientation in large scale movements of fishes such as the upstream spawning migrations of salmon (*e.g.*, Ward, 1927, 1928) makes this mode of orientation one of especial interest.

### *Responses to Chemical Stimuli*

Experiments with gustatory stimuli yielded surprising results since with the amplification ordinarily used dissolved substances in the water were found, apparently, to initiate no nerve impulses from the barbels and lips. Acetic acid solutions ranging in concentrations from 1.0 per cent to 20 per cent, a 10 per cent solution of NaCl, and a saturated solution of sugar were used as stimuli. In addition juice pressed from meat which normal catfish devour voraciously was tested. These solutions were allowed to diffuse very slowly against immersed lips and barbels which before, during, and after chemical stimulation were highly responsive to mechanical stimuli. By adjusting the potentiometer controlling the sensitivity of the output amplifier to yield maximum sensitivity, very indistinct impulses were visible on the screen in response to chemical stimulation against the background of a very ragged base line. Photographs of these impulses, however, were not very convincing since the camera speed was slow compared to that of the standing wave screen and the impulses were largely ob-

<sup>1</sup> Loeb (1918) did not apply the notion of bilateral tactile stimulation as a basis for rheotropism. He preferred to explain the upstream orientation of fishes in terms of movements of retinal images in the opposite direction to that of progression—a sort of nystagmus phenomenon. In cases of “treadmill” rheotropism in which swimming takes place without forward progression, no movements of retinal images occur. While the movement of retinal images undoubtedly explains certain cases of apparent rheotropism (Lyon, 1904, 1905, 1909) it is certainly only a partial explanation.

literated by the fluctuation of the base line. The fact that impulses of extremely low potential were being initiated was best indicated by the loud speaker since characteristic sounds were produced lasting sometimes as much as several minutes after exposure of the receptors to all of the above solutions with the exception of the sugar solution. The sounds were not produced by tap water or by Ringer's solution—they were renewed by reexposure to the stimulus, except in cases of exposures to very strong acid (concentrations  $> 5$  per cent). This treatment evidently anesthetized or destroyed the taste-buds after about a minute's exposure.

The striking thing about these results is that they indicate a high degree of quantitative specificity of impulses for taste in *Ameiurus nebulosus* Les. The amplitude of the impulses for taste are at the very threshold of sensitivity for present recording systems; they are of the order of 5 microvolts. They show roughly less than 10 per cent of the amplitude of the impulses initiated in different fibers of the same nerve in response to mechanical stimulation.

Since, in general, small action potentials are produced by small fibers and large action potentials by large fibers, one is led to suspect that fiber size may be the basis of the impulse specificity in this case. Herrick (1901) pointed out that the mandibular and maxillary branches of the trigeminus both receive general cutaneous and communis fibers in approximately equal proportions and furnish all of the barbels with fibers from these two systems. In *Ameiurus*, as indeed in siluroids and cyprinoids in general, the taste-buds are primarily innervated by the communis components of the 7th cranial nerve and to a lesser extent by components of the 9th and 10th cranial nerves. The communis fibers to the taste-buds take origin from the geniculate ganglion. The tactile endings in *Ameiurus* are of the free-ending type and are supplied by fibers from the Gasserian ganglion. To quote from Herrick (1901, p. 183): The cells of "the geniculate ganglion are all small, so that the general relations of the two ganglia can be determined despite their intimate fusion." This difference in size of the cells of origin which send axons to the taste-buds and to tactile endings respectively may therefore serve as a basis for the specificity in magnitude of potential of the impulses for the two sense modalities.

## SUMMARY

1. Receptors in the lips and barbels of the catfish *Ameiurus nebulosus* Les. are very sensitive to mechanical stimuli, giving large rapid (*A*-type) impulses in fibers of the facial nerve in response to touching the receptive surfaces and to movements of the water in which the preparation is immersed.

2. The great sensitivity of the barbels and lips to currents of water and the bilateral symmetry of the distribution of sensitivity of the facial nerve may serve as a basis for observed rheotropic orientation in the catfish.

3. Acetic acid, NaCl, and meat juice, dissolved in the water bathing the barbels and lips, set up impulses of very small and barely detectable potential in the fibers of the facial nerve.

4. It is suggested that the specificity of impulses for the two sense modalities may be correlated with the large size of the cells of origin of the axons in the Gasserian ganglion supplying tactile receptors and the small size of the cells of origin in the geniculate ganglion sending axons to taste-buds.

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# ELECTRICAL RESPONSES FROM THE LATERAL-LINE NERVES OF CATFISH. I

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## INTRODUCTORY

The function of the lateral-line organs of water-inhabiting vertebrates has been a subject of interest to biologists for many years. Externally the lateral-line system is marked in fishes by rows of pores. The main lateral-line canal extends typically along the flank from head to tail and is a conspicuous marking on most fishes. In the head this canal branches into three stems, one of which passes forward above the eye, another forward and below the eye, and a third downward and over the lower jaw. Lying between the pores in the canal are sense organs composed typically of supporting cells and sensory cells. In the catfish *Ameiurus nebulosus* Les., the animal used in the following experiments, the receptive cells (neuromasts) lie close to the lumen of the canal, and at their distal ends very fine hair-like processes extend into the lumen (Brockelbank, 1925). The receptive cells are supplied by the lateral-line nerve which runs along below the canal. The neuroanatomy of the lateral-line system in *Ameiurus* has been described by Herrick (1901). The organs of the lateral-line canal are supplied by the vagus nerve which has terminations in the tuberculum acusticum. The structures in the catfish are fairly typical of those in other fishes.

Fuchs in 1895 investigated the functions of the organs by recording action currents from the lateral-line nerves of the torpedo fish. He obtained negative deflections of his galvanometer from what he attributed to be action currents of the lateral-line nerve in response to tactual stimulation of the end organs. He failed to get responses to chemical stimulation or to changes in temperature. From these experiments he concluded that the lateral-line organs are tactile receptors.

Parker (1902, 1904) reviewed the literature concerning the function of the lateral-line organs and presented experiments describing the behavior of normal fishes and

those deprived of functional lateral-line organs in the presence of various types of stimulation. The experiments were carried out systematically on *Fundulus* and confirmed with some seven other species of fish. Parker concluded that these organs are mechanoreceptors responding to low vibration frequencies in the water (six per second), and that they are not stimulated by light, heat, salinity of water, food, oxygen, carbon dioxide, foulness of water, water pressure, water currents, or sound.

In 1917 Parker and Van Heusen published a report on the mechanical stimulation of the catfish *Ameiurus nebulosus*. Responses of groups of catfish were examined after operations which rendered certain specific sense organs non-functional. There were eight groups of fishes ranging from those in which the three sets of possible mechanoreceptors—skin, ears, and lateral-line organs—were functional, through those in which any two or only one of these sets of organs were functional. One group was also examined in which all of the three types of receptors had been eliminated.

The skin of *Ameiurus* was found to be stimulated by the dropping of water, by water currents, by a slow vibratory movement of the whole body of water, by the impact of a leaden ball on the end of the aquarium, and by low tones of a submerged telephone up to 172 double vibrations per second. The ear of *Ameiurus* was found to be stimulated by slow vibratory movements of the whole body of water, by the impact of a leaden ball on the end of the aquarium, by a whistle blown in the air, and by telephone vibrations ranging roughly between 43 and 2700 double vibrations per second. Dropping of water and water currents did not stimulate the ears. The responses initiated from stimulating both ears and skin were locomotory. Fishes in which the auditory nerves had been cut and the skin anesthetized, but which maintained functional lateral-line organs, were found not to respond positively to any of the above stimuli. However, the receptivity of the lateral-line organs to certain types of stimuli was clearly seen in those groups of fishes in which the lateral-line organs were functional along with either the ears or the skin. In these groups the lateral-line organs seemed to inhibit responses normally initiated by stimulating the ears or the skin. Thus, in response to blows from a leaden ball on the end of the aquarium, when only the skin was intact, the percentage of responses was 27, but when both skin and lateral-line organs were effective the response fell to 11 per cent. When only the ear was intact the percentage of responses was 73, but when the ear was combined with the lateral-line organs the percentage dropped to 33. Probable errors were not computed, but owing to the numbers of observations these differences are evidently significant. The presence of lateral-line organs in both cases markedly reduced the percentage of responses. From their experiments Parker and Van Heusen concluded that the lateral-line organs of *Ameiurus* respond to impacts of a leaden ball on the walls of the aquarium, to slow vibratory movements of the whole body of water, to the lower tones of a submerged telephone, up to approximately 344 vibrations, but not to a whistle blown in the air, to the dropping of water, to currents of water, or to the higher tones of a telephone. The responses to the

stimuli were found to be inhibitions of those responses initiated through the skin and the ears.

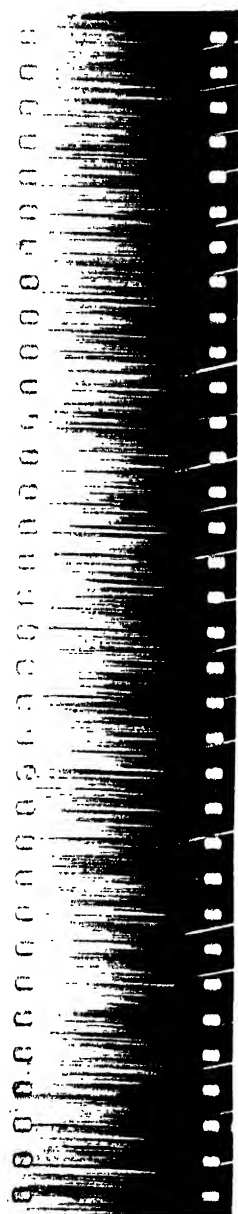
In view of the inhibitory action of the lateral-line organs, it occurred to the writer that an examination of the electrical responses of the lateral-line nerve of the catfish not only might shed light on the normal functioning of the lateral-line receptors, but might also furnish a clue as to the possible nature of the inhibitory processes involved.

#### EXPERIMENTAL

In the present experiments only the lateral-line organs in the side of the fish were studied; the organs of this system located in the head were not examined. Fishes were prepared for the experiments by cutting through the medulla with a knife. In some experiments the entire head was removed, in others cordotomy was performed so as to leave the circulation relatively intact. The nerve supplying the lateral-line organs was bared for about 1 cm. by removing the skin and covering tissues over the lateral line streak just anterior to the dorsal fin. After careful freeing, the nerve was tied with a thread and cut at its proximal end. In this way a length of nerve was obtained which remained connected with the lateral-line organs of the posterior part of the fish's body. The fish was then immersed in water so as to cover the organs to be tested. In some experiments it was laid with its operated side uppermost in a glass dish with water coming up just to the incision; in others it was suspended tail down in a jar of water. The nerve, moistened with Ringer's solution, was drawn by the thread across silver, silver-chloride electrodes connected to the recording system.

The recording system consists of an eight-valve, resistance-capacity coupled amplifier which operates an iron armature oscillograph (Matthews, 1928). Some of the amplified action potential from the nerve was further increased by a subsidiary amplifier and recorded as sound on a loud speaker. Light from an arc lamp, partially intercepted by a stationary straight edge, is reflected from the oscillograph mirror into a camera, where motion of the oscillograph tongue upon which the mirror is mounted is recorded on moving bromide paper. Another portion of the light reflected from the oscillograph mirror is projected by rotating mirrors as a standing wave on a screen. In this way the experimenter can hear the amplified impulses from the nerve, see them on a screen, and photograph them when he so desires.

With the first lateral-line nerve investigated a curious phenomenon was apparent. Instead of the usual smooth base line with nerve impulses showing up as a result of specific end organ stimulation, great activity was spontaneously manifested from the supposedly resting lateral-line system. The system was found to be continuously emitting nerve impulses at a high frequency, each nerve fiber apparently dis-

*a*

Electrophysiological recording from lateral-line nerve of catfish

*b*

↔ 0.1 second

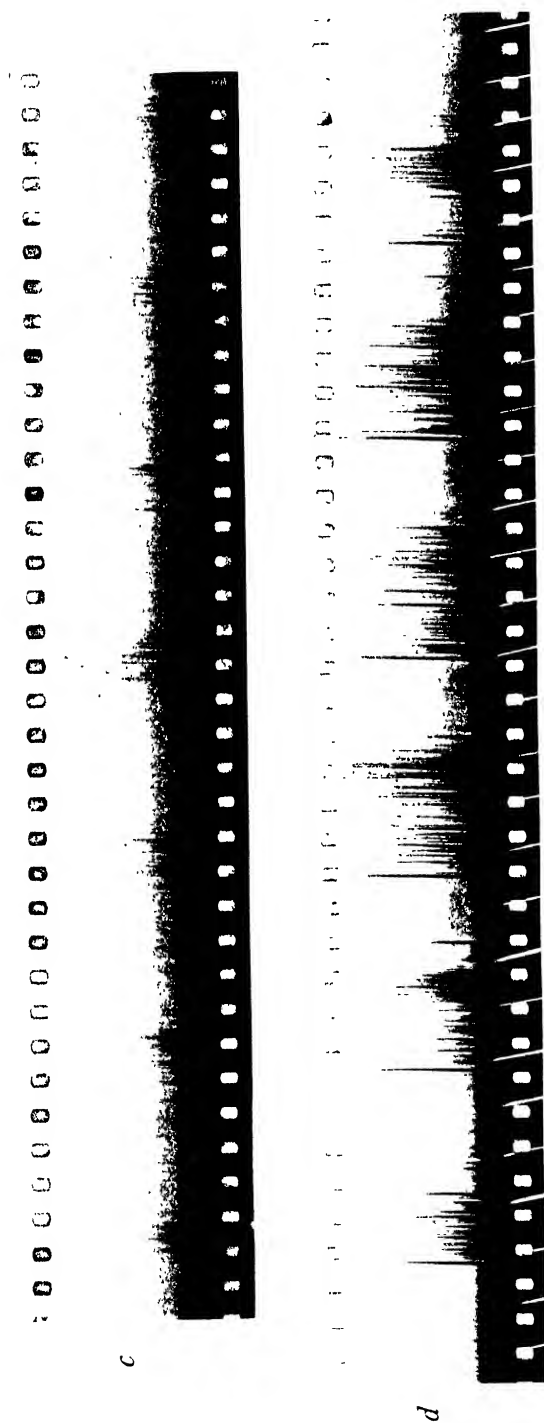


FIG. 1. Responses of catfish nerves; time marked in tenths of a second. The length of the spikes is proportional to the action potentials of the nerve fibers.

(a) Spontaneous firing of a lateral-line nerve.

(b) Base line for nerve used in making Fig. 1a after crushing the nerve between the electrodes and its entrance to the body.

(c) Responses from a spinal nerve when the skin supplied by it is stroked by a feather. The bursts of impulses mark the stroke.

(d) Responses from a branch of a facial nerve supplying sense organs in the lower lip when the lip is stroked with a feather.

charging at a rhythm of its own independently of the other fibers. At first it seemed possible that this activity might be due either to an artifact of the apparatus or to injury of the nerve in the course of the operation. Control experiments show that these explanations are not tenable. The lateral-line system in *Ameiurus* normally appears to be in a state of spontaneous activity, and its specific responses to stimuli take place against this background of activity. Out of thirty-two nerves examined all but three were found to be spontaneously discharging. These three nerves, however, failed to show any response to stimulation of the lateral-line organs. The same stimulation produced characteristic responses from the twenty-nine spontaneously active nerves. It seems probable, therefore, that these three nerves were completely non-functional, due perhaps to injury from the operation.

Fig. 1*a* shows a typical lateral-line nerve firing spontaneously. This sort of activity goes on unabated throughout an experiment. In some cases there was no reduction in the spontaneous discharge several hours after the first record was made. The impulses are typically diphasic, although they can be made monophasic by crushing the nerve under the distal electrode.

That we have to do with nerve impulses and not with artifacts of the apparatus is further borne out by the fact that reducing the temperature of the preparation produces a marked decline in the frequency of the spontaneous discharge and of the rate of the conduction of the impulses (*cf.* Fig. 4). Cutting the nerve where it enters the body but leaving it on the electrodes and in physical contact with the body produces an immediate cessation of the spontaneous activity. The nerve is also silenced by anesthetics placed either on the nerve or on the end organs. Water on the exposed nerve also causes failure of the spontaneous discharge. Fig. 1*b* shows a typical smooth base line after cutting the nerve between its region of entrance to the body and the electrodes. This nerve is the same one used a few minutes previously for obtaining Fig. 1*a*. Sometimes failure of activity of the nerve occurred during an experiment. Drawing the nerve further along the electrodes frequently was sufficient to renew the discharge. Cutting the lateral-line nerve and canal midway between the original incision and the caudal end of the line of pores furthermore resulted in a substantial reduction of the density of the discharge due to the severance of connection of the neuromasts posterior to this second incision.

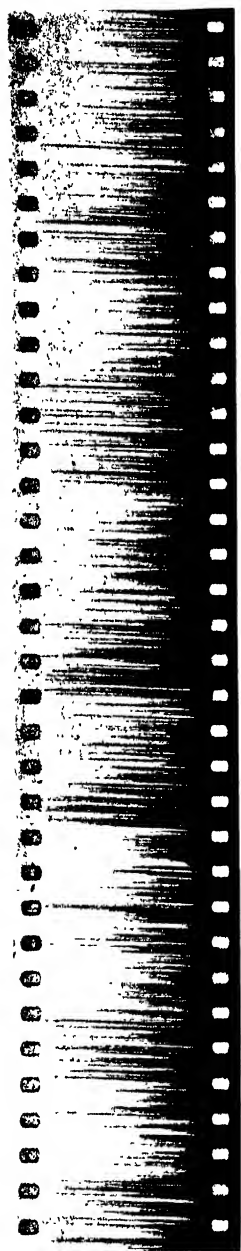
That the spontaneous activity is not a product of the operation is indicated by the last fact. Furthermore, rough treatment of the nerve, such as pulling, permanently stops all responses. The nerve lies just under the skin at the place of incision and may be cleanly dissected free without difficulty. Since it is cut at the anterior end of its freed length, it is not in connection with the central nervous system. Anatomically there is no reason to believe that the nerve distal to the electrodes makes connection with the spinal cord. The independence of the spontaneous activity from possible connections with discharging internuncial neurones of the cord was checked by pithing the cord. This operation has no effect on the activity.

Other nerves were examined in *Ameiurus*, but none were found to yield impulses spontaneously. The body cavities of several fishes were opened and spinal nerves were tested. Fig. 1*c* shows a typical response of a spinal nerve to touching with a feather the highly localized area of skin which it supplies. The base line is smooth. The nerve is only active in response to direct stimulation of the end organs. Fig. 1*d* shows typical responses obtained from a branch of the facial nerve supplying the lip and base of the maxillary barbel when the skin is lightly touched (*cf.* Hoagland, 1932: 33). The smooth base line is apparent.

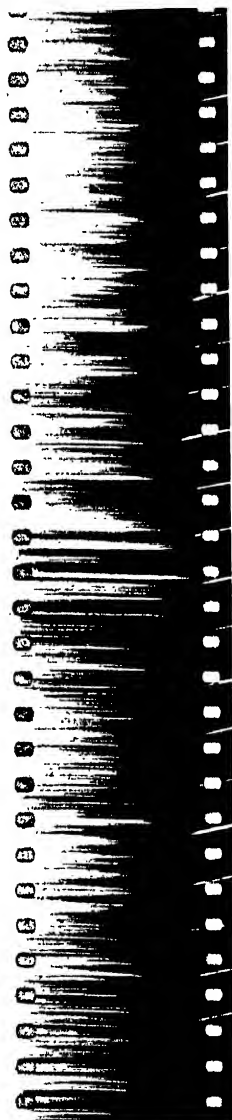
Changes in the frequency of the spontaneous discharge of the lateral-line nerve were found to be brought about by two kinds of stimuli: (1) mechanical stimuli; (2) temperature changes. No responses were elicited by chemical stimulation.

Lightly stroking the skin over the lateral-line organs was found to produce a marked increase in the frequency of impulses during the stroking. Fig. 2*a* shows a spontaneously firing lateral-line nerve and the increased density of discharge when the flank over the lateral-line is stroked with the tip of a feather. These responses were especially audible on the loud speaker. This finding is consistent with that of Fuchs (1895) who, however, records no evidence of spontaneous discharge from the nerve. His results are to be expected in view of the comparatively crude galvanometers then available which would not be able to separate the impulses due to single fibers. If the lateral-line system operates in the torpedo fish as it does in the catfish, continuous activity of the nerve would merely serve to set what would appear to be a stable base line for a relatively insensitive recording system. Only





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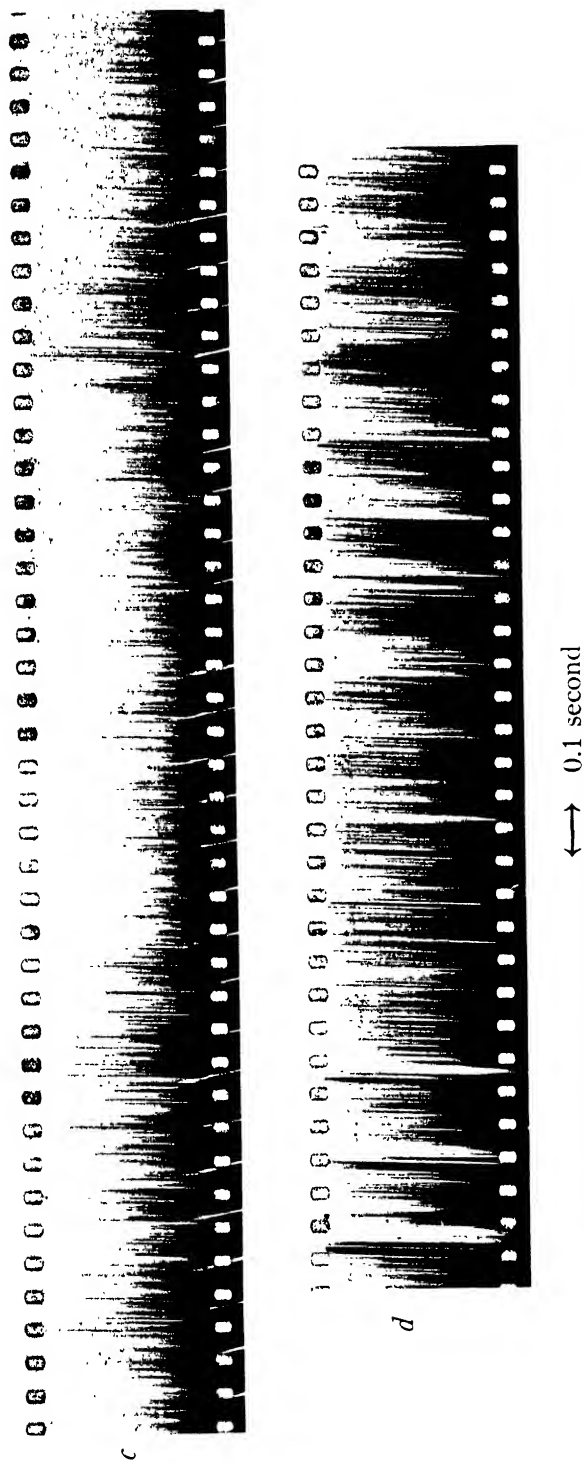


FIG. 2. Responses of a lateral-line nerve to various mechanical stimuli.

- (a) Responses of a lateral-line nerve to stroking the skin above the canal with the tip of a feather. An increased density of discharge marks each stroke.
- (b) Effect of squirting a stream of water from a pipette against the lateral-line.
- (c) Increase in density of discharge accompanying spinal swimming movements.
- (d) Effects of rippling the surface of the water which covers the lateral-line canal.

Effects of two such movements are recorded. Time in tenths of a second.

relatively gross changes of potential would cause a deflection from this base line.

Streams of water directed against the lateral-line from a pipette also produced an increase in the responses similar to those due to direct stroking (Fig. 2*b*).

The presence or absence of water over the lateral-line organs seemed to make no difference in the frequency of the spontaneous discharge. This was tested by recording the responses both before and after immersion of the flank of the fish in water and in some cases after subsequent siphoning off of the water. Prolonged drying of the skin ultimately produced a permanent cessation of all activity from the nerve.

Flexion of the body of the fish by means of a thread around its tail usually produced an increase in the density of discharge during the time of bending. In some preparations slow, spontaneous spinal swimming reflexes persisted when the body was immersed in water. These rhythmic movements often produced small additional bursts of impulses clearly audible on the loud speaker from the spontaneously firing neuromasts. Fig. 2*c* shows responses resulting from reflex swimming movements. The upper part of the trunk was stationary and the nerve did not move on the electrodes. It is probable that pressure from the surrounding tissues is the stimulus increasing the discharge of the neuromasts when the body is bent.

Fig. 2*d* shows responses to rippling the water with a feather.

The response to vibratory stimuli was peculiar. Three tuning forks were used, giving respectively 100, 200, 250 double vibrations per second. These are within the range of frequencies which Parker found would stimulate the lateral-line organs of *Ameiurus*. Fig. 3*a* shows the response of a lateral-line organ to an electrically earthed tuning-fork of 200 vibrations after setting it in motion and pressing its shank against the outside wall of the glass vessel containing the preparation. The fibers of the lateral-line nerve which had previously been firing independently of each other now become synchronized and beat in a rhythm of their own, in this case about 40 beats per second. This rhythm seems to be characteristic of the tissue and is independent of the frequency of the tuning-fork, since frequencies of 100 or of 250 brought about the same frequency of beating as the fork with the

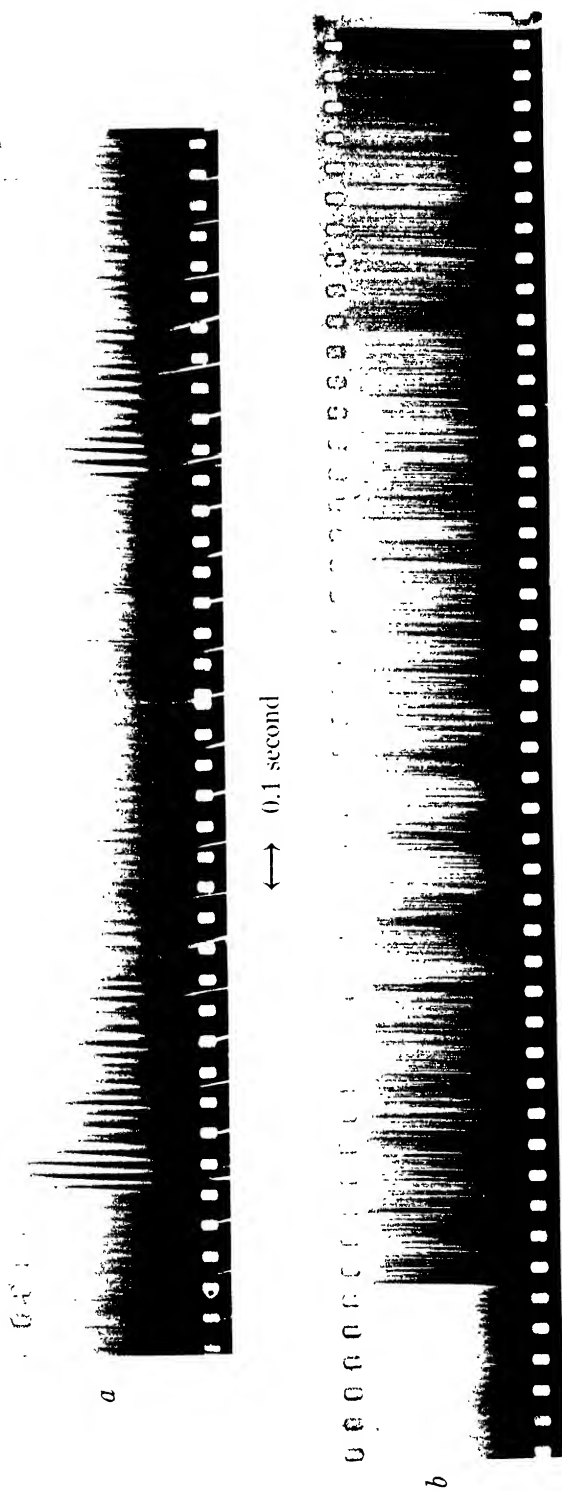


FIG. 3. (a) Synchronized rhythmic discharge of a spontaneously active lateral-line nerve at 40 per second when a vibrating tuning-fork giving 200 double vibrations per second is twice pressed against the outside of the vessel containing the preparation.

(b) Responses to pressure on skin receptors of a frog from a dorsal cutaneous nerve. The frog was placed in the same experimental setting as the catfish from which record (a) was obtained. The vibrating tuning-fork was pressed against the outside of the containing vessel as above, but no synchronous responses occurred.

frequency of 200. Only approximately 60 per cent of the preparations showed this response to tuning-forks. The remaining 40 per cent were unresponsive to the forks, but were responsive to other forms of mechanical stimulation.

To be sure that the beating of the lateral-line system is a function of the tissue and is not an artifact of the apparatus, certain control experiments were necessary. Mechanical changes, such as vigorously tapping the containing vessel, will produce sudden shifts in the base line with high amplification, which, while not especially resembling nerve impulses in form, may nevertheless be misleading. Lateral-line nerves were crushed and their spontaneous activity stopped. A tuning-fork was then set vibrating and pressed against the outside of the dish containing the preparation. No such effects were recorded as those seen in Fig. 3*a*, although sometimes slight disturbances were superimposed on the smooth base line which corresponded identically with the frequency of the tuning-fork. In fact these disturbances were photographed and used in checking the calibration of the forks. This effect was very different from that recorded in Fig. 3*a*.

The fact that translation of the mechanical energy of the fork into electrical disturbances did occur in the system and was transmitted to the electrodes through quite inactive nerve (a Ringer-moistened thread in contact with the fish's body would give the same result) made it seem possible that this disturbance, superimposed on the background of spontaneous activity of the normal nerve, might produce a periodic activity of the recording system, and thus account for the result seen in Fig. 3*a*. To examine this possibility it was necessary to find a nerve which could be made, by suitable stimulation, to give a discharge density similar to that of the lateral-line nerve. Such nerves could then be tested under the same conditions as those used in testing the lateral-lines nerves. The most suitable preparations for this purpose turned out to be the dorsal cutaneous nerves of frogs. A frog, with brain and cord pithed, was put in the same dish used for the experiments with the catfish. The level of the water was brought up so as to immerse all but the back of the frog lying ventrum down. The nerve, cut at its entrance to the cord, was drawn across the electrodes.

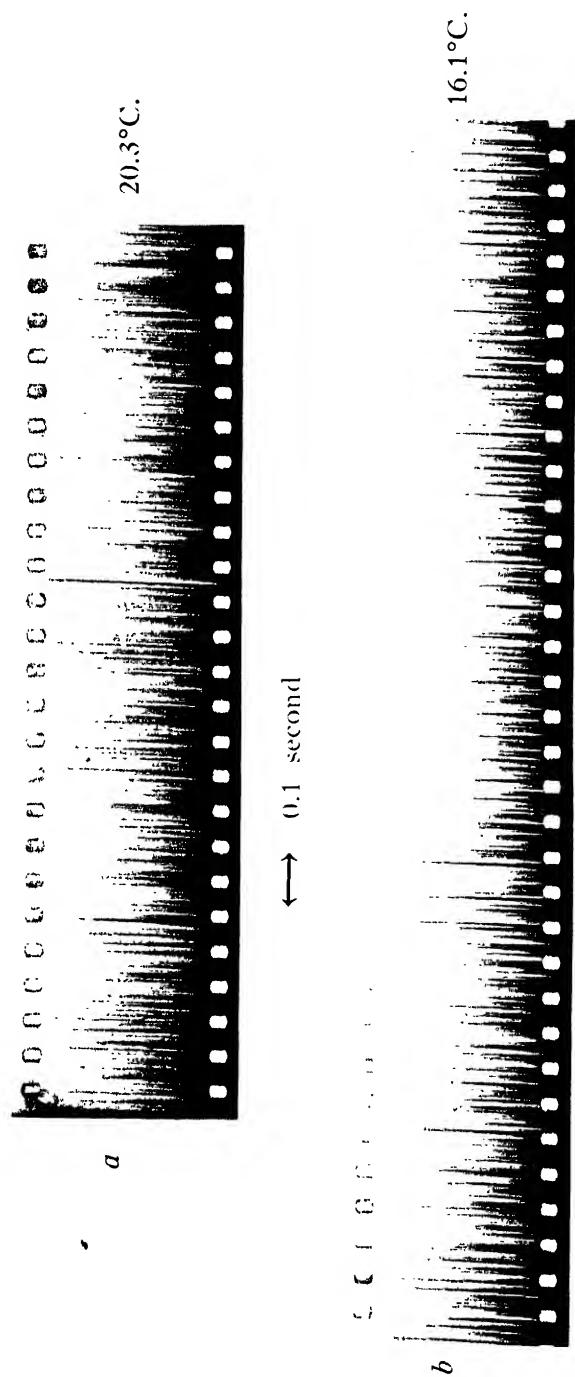
Fig. 3*b* shows a typical discharge initiated by pressing a frog's skin with a feather held in the hand. The vibrating tuning-fork (200

double vibrations) was pressed on the outside of the dish near the beginning of this discharge. No response such as is shown in Fig. 3*a* was found to occur in repeated experiments involving twelve dorsal cutaneous nerves. Since the experimental set-up and the massiveness of the discharge is the same in the experiments with the catfish and those with the frog, it seems safe to conclude that the phasic effects shown in Fig. 3*a* are produced by the rhythmic beating of the lateral-line end organs brought into "step" with each other by the vibratory stimulus.<sup>1</sup>

From the foregoing experiments the lateral-line organs appear to be tactile receptors, a finding quite in agreement with much of the previous work, especially that of Parker. The peculiar thing about these organs is that they respond to pressure of moderate durations by an increase in frequency. For short rhythmic pulsations of pressure as produced by tuning-forks the randomly beating neuromasts get into phase with each other and beat synchronously at frequencies ranging from 20 to 70 beats per second, depending upon the individual animal, but independent of the frequency of the stimulus for forks giving 100, 200, and 250 double vibrations.

In view of the continuous activity of the lateral-line system it seemed probable that temperature might modify the activity. Despite the negative findings of both Fuchs and Parker, such modification might serve as a basis for temperature discrimination. Tests of the spontaneous activity of the same lateral-line nerve at different temperatures consistently showed marked changes in frequency with temperature. For these experiments a decapitated fish was hung tail downward in a glass jar containing water. The level of the water was adjusted so as just to cover the body up to the incision. As in all of the experiments, care was taken to keep the wound, and especially the nerve, moistened with Ringer solution. The temperature of the water in the jar was read by means of a thermometer suspended in it, and the discharge of

<sup>1</sup> In some control experiments a massive discharge of impulses was produced by putting 10 per cent acetic acid on the frog's skin. The frog gastrocnemius was also stretched and the discharge of impulses was recorded from the sciatic nerve. Here again rhythmic effects such as are seen in Fig. 3*a* were never produced when the tuning-forks were applied, during the discharge, to the outside of the dish containing the preparation.



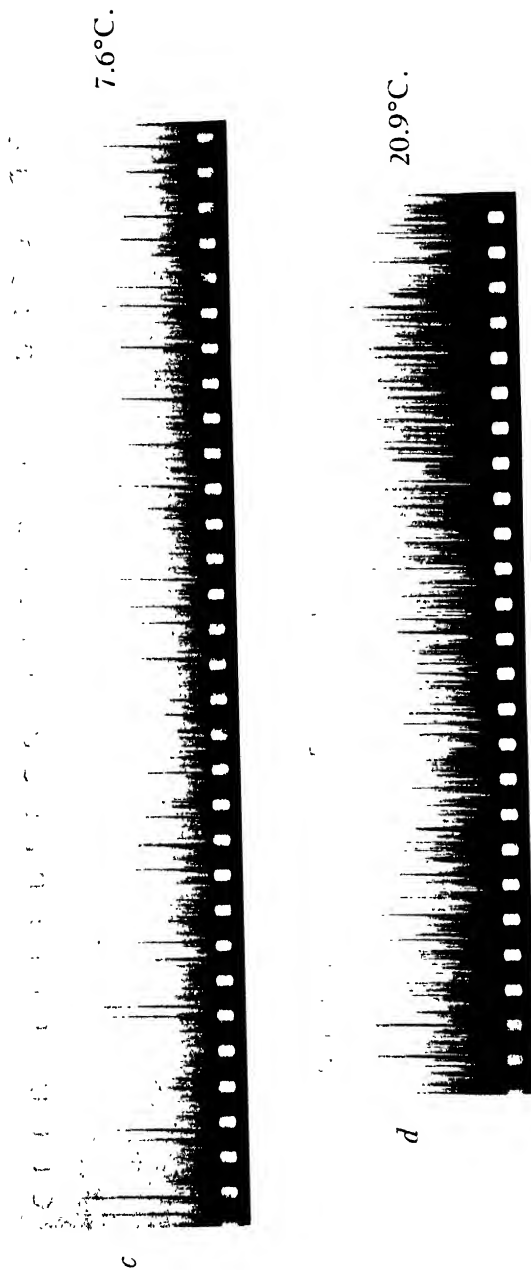


FIG. 4. Spontaneous discharge of a lateral-line nerve at different temperatures.



the nerve was photographed. In addition to the spontaneous discharge, the effects of certain of the mechanical stimuli which have just been discussed were also measured. The reception of these stimuli seemed to be unaffected by temperatures ranging between 5° and 28°C. Data were obtained on the responses of ten lateral-line nerves for this range of temperatures. As soon as the suitable record had been obtained for a given temperature, the water in the jar was quickly siphoned off without moving the preparation, and was replaced by water at another temperature. This change of water could be effected in 30 seconds. A series of beakers with water at suitable temperatures was kept on hand to facilitate rapid changes of the bath. Measurements of the discharge at the new temperature were made after approximately 1 minute.

Fig. 4 shows typical spontaneous responses of a lateral-line nerve at four different temperatures.<sup>2</sup> It may be seen that the frequency declines considerably when the temperature is lowered, and that it recovers fully on raising the temperature again. A quantitative analysis of the effect of temperature on the frequency of discharge is in preparation. The results are in disagreement with those of Fuchs reported in 1895, who was unable to obtain responses to changes in temperature from the lateral-line nerves of the torpedo fish. Positive results with the electrical recording apparatus then available would be unlikely if the responses to temperature changes are similar in the lateral-line nerve of the skate and the catfish. A decline in frequency with temperature would merely shift slightly the base line of an insensitive instrument, a change the significance of which might readily escape attention.

To test the possibility that the lateral-line system may be concerned with temperature reception, I endeavored to obtain impulses in response to temperature changes from spinal nerves and from branches to the facial nerves in the catfish. Marked temperature changes between the ranges of 0°C. (from ice on the skin) and 28°C. produced no nerve impulses. Some six spinal nerves chosen at random were tested

<sup>2</sup> By severing the lateral-line system just caudal to the junction of the nerve to the body it has recently been possible to get responses from only one organ supplied by 10-15 fibers. By chilling this preparation it is possible to silence progressively all but one fiber.

in two fishes, and branches of the facial nerves supplying in different cases the lips and barbels in three fishes were examined. The areas supplied by the nerves were in all cases carefully mapped by touching the skin with a feather and listening to the burst of impulses on the loud speaker. Water from a pipette at temperatures above or below that of the water bathing the skin was allowed to diffuse slowly onto the sensitive area. A few responses were often initially produced owing to the pressure of the diffusing water, but these stopped with the current, and were the same regardless of the temperature of the water. This negative evidence strengthens the possibility that temperature sensitivity in the catfish may be mediated by the lateral-line system which alters the frequency of its spontaneous discharge with temperature. The position of the neuromasts just under the skin, their wide distribution, not only along the lateral-line, but in the head as well, is anatomically consistent with their use as temperature receptors.

#### DISCUSSION

The strongest evidence against the notion of the lateral-line organs functioning as temperature receptors was presented by Parker's (1904) experiments. The behavior of *Fundulus* in which the lateral-line organs were non-functional was indistinguishable from that of normal fishes, at various temperatures, and Parker concluded that heat is not a stimulus to the lateral-line organs.

These results, however, do not seem to me to be conclusive. The behavior of all the fishes at any temperature was at first to go to the bottom of the aquarium and to remain there for varying periods of time. This seems to have been a sort of escape movement initiated by placing them in the vessel. During these intervals at the bottom the fish would approach the temperature of its surroundings, and its subsequent rise to the surface at certain temperatures might depend entirely upon factors conditioned by the internal temperature, independently of the activity of temperature receptors which the fish either might or might not possess. This criticism may be applied to many studies of the effect of temperature on cold-blooded vertebrates where one is concerned primarily with the problem of thermal receptors.

Certain observations, however, do indicate that fishes possess a temperature sense. The influence of temperature in determining the migratory course of sal-

mon to inland spawning waters has been pointed out by Ward (1927, 1929). The fishes swim upstream against the current, and when they come to branching streams they always swim into the colder of the two, other things being equal.

Wells (1914) has reported experiments on a variety of species of fresh water fish including the catfish. His work indicates that fishes possess a high degree of sensitivity to temperature. Fishes were put in a long, narrow tank, with water at different temperatures flowing in at the two ends. The water flowed to the middle of the tank and thence out through drains at the top and bottom. The result was a mixing of the water and the production of a temperature gradient, especially in the middle third of the tank. The fish was introduced at the center of the tank, and a graph of its movements was made. In a like manner control experiments were made using the inflowing water at both ends of the tank at the same temperature. In the control experiments the movements were of a random nature, but in the experiments with different temperatures at the two ends, all four species of fish examined, including *Ameiurus melas*, turned sharply away from the cold portions of the tank and elected to remain in water of 16–19°C. According to Wells, fishes react to variations of temperature as small as 0.1°C.

The present findings, while not proving that the lateral-line organs act as thermal receptors, do show that they may do so, since they respond to differences of temperature which other cutaneous organs apparently do not.

According to Herrick (1901) the lateral-line sense organs of *Ameiurus* are of three structural types: (1) large pit organs; (2) small pit organs; (3) canal organs. The pit organs are scattered over the skin of the flank, and are supplied by twigs of the lateral-line nerve. It is possible that one or another of these types of organs may alone be composed of direct tactile receptors, and that another type may function as spontaneously beating receptors. That this may be true is suggested by Fig. 2, especially by records (a) and (c). Here mechanical stimulation increases not only the frequency of discharge but also raises the height of the potential spikes. This could be brought about by the simultaneous discharge from several fibers or by bringing into play entirely new receptors whose nerve fibers give large action potentials. From the single appearance of the potential spikes obtained on stimulation this latter alternative seems likely. On the other hand, the increased height of the potential spikes of Fig. 3 in response to tuning-fork stimulation is apparently due to the synchronizing and summation of the discharges of the spontaneously firing fibers.

The continuous discharge of the neuromasts would hardly be ex-

pected from their structure. It is possible, however, that the hair-like filaments of the sensory cells projecting into the lumen of the canal, may actually be cilia which by beating set up a state of continuous excitability in the end organs of which they are a part. I know of no direct evidence concerning this matter, one way or the other.

The action of the lateral-line system of *Ameiurus* in inhibiting responses normally induced by mechanical stimulation of the ears and skin (Parker and Van Heusen, 1917) bears certain points of resemblance to the inhibition of tactile receptivity by the visual apparatus as described by Crozier (1918). Crozier found that the hamlet or grouper fish, *Epinephelus striatus* (Bloch), undergoes a marked increase in sensitivity to slight mechanical disturbances in the water after the exclusion of vision. The fishes were able to avoid collisions with rods and wires in an aquarium much more effectually after depriving them of vision than with the visual apparatus functioning. The visual apparatus inhibits the reception by the skin of mechanical disturbances produced by objects in the water—objects which may either be moving toward the fish or towards which the fish moves. The continuous stream of centripetal impulses from the retina evidently produces central inhibition in the centers concerned with the reception of tactual cues. The lateral-line nerve, in a state of constant spontaneous activity, produces a centripetal stream of impulses which may be compared to the impulses continuously passing over the optic nerve when light acts on the retina. The centripetal impulses from the lateral-line nerve may also produce central inhibition of incoming tactual and auditory impulses in the way that the visual impulses centrally inhibit those from the tactile receptors in *Epinephelus*. The inhibitory effects of the lateral-line system may therefore be a corollary of the spontaneous activity.

The responses of the lateral-line organs to movements of the fish's trunk are interesting, since they may serve as kinesthetic cues in swimming. These impulses may act to regulate speed of swimming and direction of turning, especially in darkness and when the fish is in midwater.

#### SUMMARY

1. Records of impulses from the lateral-line nerves of catfish show that the lateral-line organs are in a state of continuous activity, producing a massive discharge of impulses.

2. The discharge may be increased during the direct application of pressure on the skin over the lateral-line canal, by ripples in the water, by irregular currents of water, and by movements of the fish's trunk.

3. The asynchronously discharging lateral-line organs respond to

vibratory stimuli from tuning-forks by getting into phase with each other and by beating synchronously at frequencies ranging from 20 to 70 per second. The frequency of beating for a given preparation is independent of the frequency of the tuning-fork for the fork frequencies of 100, 200, and 250 double vibrations which were used.

4. The continuous discharge of the lateral-line system is markedly changed by alteration of temperature. The frequency declines on lowering the temperature and rises on increasing it. Spinal and facial nerves in the catfish fail to yield nerve impulses in response to changes of the skin temperature between 0° and 28°C., although the intact animal is known to be sensitive to temperature differences.

5. The action of the lateral-line system of *Ameiurus* in inhibiting responses initiated through the skin and ears (Parker and Van Heusen, 1917) is discussed in the light of the present experiments.

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# QUANTITATIVE ANALYSIS OF RESPONSES FROM LATERAL-LINE NERVES OF FISHES. II

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## I

In a recent paper (Hoagland, 1932-33) it was pointed out that the lateral-line nerves of catfish *Ameiurus nebulosus* appear to be normally in a state of continuous activity, discharging impulses at a high frequency. These impulses were shown to be initiated by the sense organs (neuromasts) of the lateral-line system. The frequency of the discharge could be modified by various forms of mechanical stimulation and by altering the temperature of the receptors. The present paper is based upon a quantitative study of the effect of temperature on the impulse discharge from the lateral-line organs of the catfish, and also an analysis of the contribution which the individual receptors make to the total discharge in the nerve.

## II

Tests of lateral-line nerves of a number of species of fresh water fish have recently been made, using the method previously described (Hoagland, 1932-33). In brief, this consists in baring the lateral-line nerve about a centimeter behind the head and dissecting it free for 1 or 2 cm. It is then tied, cut cephalad, and the freed length is drawn across silver, silver-chloride electrodes connected to the recording system. The action potentials of the nerve are amplified and recorded by an iron armature oscillograph (Matthews, 1928) used in conjunction with a camera and a standing wave screen. A loud speaker makes the amplified action potentials audible.

The lateral-line nerve of the brook trout, *Salvelinus fontinalis* (Mitchill), was also found to give a very vigorous spontaneous discharge of impulses in five out of seven nerves tested (cf. Fig. 6 *c* and *d*). Other fishes tested were: perch, four nerves; pickerel, eight nerves; blue-gilled and red-gilled sunfish, six nerves of each; *Fundulus*, five nerves; and goldfish, four nerves. No nerve impulses were obtained from the thirty-two lateral-line nerves of these other fishes. Not only were there no spontaneous impulses, but stroking the skin above the lateral-line canal and

using other forms of mechanical stimulation, effective on the catfish and trout, produced no responses.

There is an outstanding morphological difference between fishes giving responses and those which do not in the cases so far examined. Those of the former group either do not have scales (catfish) or have extremely small ones (trout), while those of the latter group are well protected by scales. The degree of myelination and mechanical strength of the very peripheral lateral-line nerves seems to be roughly an inverse function of the protection afforded by the integument. The scaly fishes examined, with the possible exception of the goldfish, have very much more delicate lateral-line nerves than have the catfish or trout, thus rendering surgical manipulation difficult. It seems probable that the more delicate nerves of the heavily scaled fishes may be damaged in the operation and thus silenced. It is also possible that shearing action of the scales while handling them may destroy the delicate connections of the nerve with the neuromasts.

### III

If one cuts through a spontaneously discharging lateral-line nerve at a point several centimeters posterior to the original incision, an immediate decline in frequency of discharge results, owing to the severance of connections with neuromasts caudal to this incision. In this way, by successively cutting the lateral-line system along the flank at decreasing distances from the region where the nerve passes from the body to the recording electrodes, it is possible to study the contribution made to the total response by the individual receptor groups located between these cuts and the initial operation. This may be done by photographing the spontaneous discharge after each fresh cut, the distance between this cut and the original incision from which the nerve emerges being carefully measured. The number of end organs remaining after each cut may be counted as follows: After completion of the experiment the fish is turned over and the opposite nerve is exposed by an operation bilaterally symmetrical to the first one. This second nerve is then pulled very gently. In the case of the catfish, and quite frequently with trout, it is possible to draw the entire nerve out of the body without breaking it. As one pulls, a puckering is detected at each neuromast group as the fibers innervating it are broken. The breaking away from the neuromasts occurs in an anterior posterior direction along the flank, so that a count of the successive puckerings gives the number of groups of neuromasts caudal to the exit of the nerve to the electrodes. By such a count the distribution of

the neuromasts as a function of the distance from the incision may be obtained. Such a direct procedure is necessary since the neuromasts in *Ameiurus* are packed with increasing closeness as one approaches the tail and are not always marked externally by pores as are those more cephalad. Control tests showed a high degree of bilateral symmetry for distribution of sense organs in a given fish, indicating that the distri-

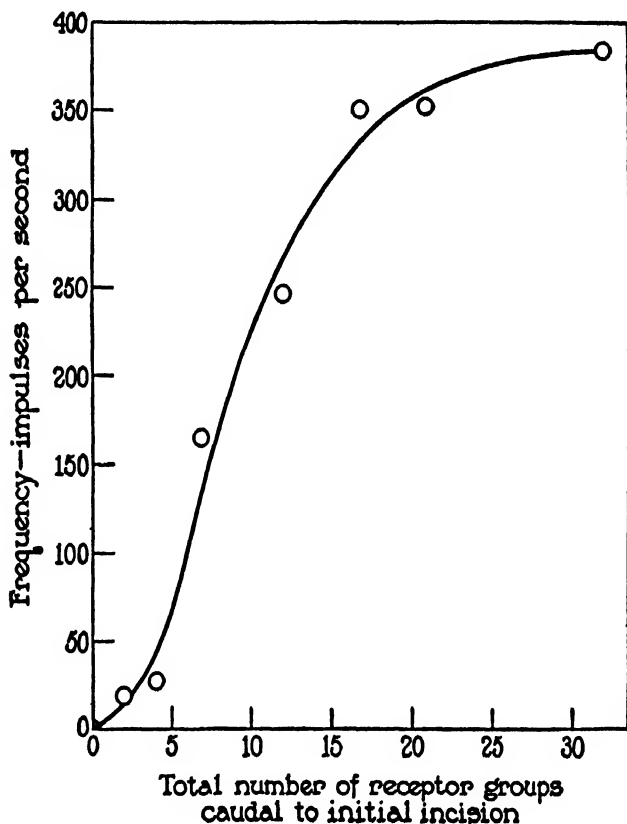


FIG. 1. Relation between frequency of nerve impulse discharge and number of contributory lateral-line receptor groups.

bution on one side could serve as a measure for that on the opposite, tested side.

Fig. 1 shows a typical curve indicating the relation between frequency of spontaneous discharge of nerve impulses from the lateral-line nerve of a catfish and the number of neuromasts caudal to the point of emergence of the nerve from the body. The curve is sigmoid,—the



flattened upper part indicating that the more densely packed organs towards the tail contribute a smaller quota of impulses to the total discharge. As will be seen more clearly presently, the curvature at

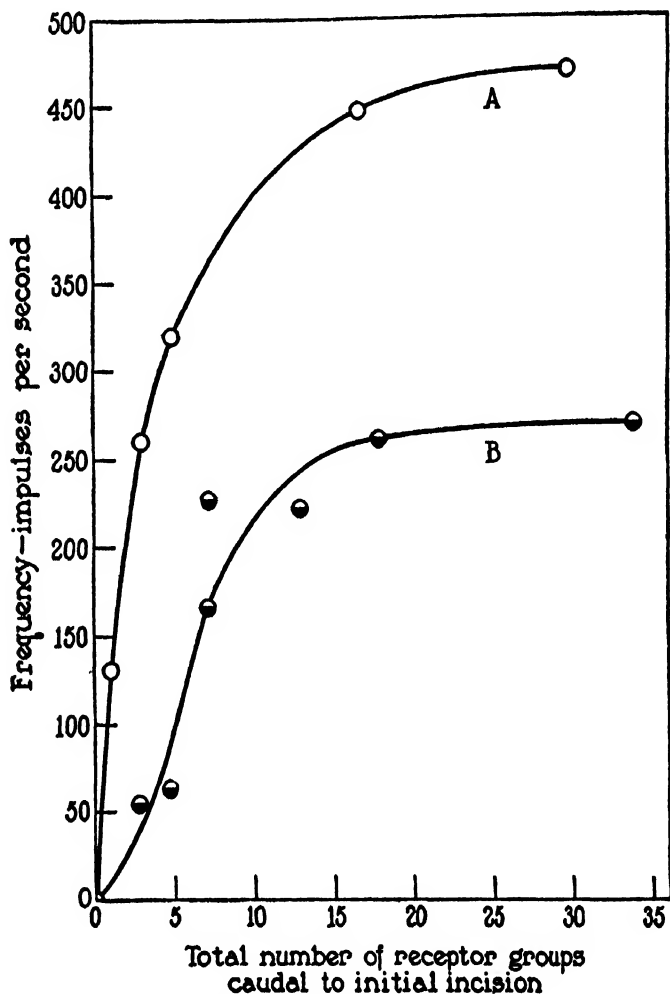


FIG. 2. Properties of the curves are discussed in the text.

the bottom of the figure may be accounted for by the fact that the first few receptor groups caudal to the initial incision are damaged by the operation, so that a full discharge from their neuromasts is not obtained.<sup>1</sup>

<sup>1</sup> The initial operation exposing the nerve involves not only cutting away a patch of skin, but also the freeing of from 1 to 2 cm. of nerve. A certain amount of

In some experiments one gets results shown by Curve A of Fig. 2, where there is an almost linear relation between frequency of impulse and the first five neuromasts posterior to the incision. This indicates, in such cases, the probable absence of damage to sense organs near the initial incision. Six out of eight experiments plotted gave sigmoid curves like those of Fig. 1. Only two curves were obtained of the type of Curve A, Fig. 2. Curve B of Fig. 2 is an example from another experiment yielding a typical sigmoid curve similar to that of Fig. 1. Unless otherwise designated the figures in this paper refer to experiments with the lateral-line nerves of catfish. The trout lateral-line nerve has not as yet been studied systematically.

Fig. 3 is the differential curve of Fig. 1, made by plotting the slope of that curve against the number of sense organ groups. It is essentially a skewed distribution curve, showing the relative frequency contributed by each of the sense organ groups caudal from the region of the initial operation.

Each point in Figs. 1 and 2 was obtained by counting the number of impulses in each of fifteen tenth-of-a-second intervals on the photographs in which 0.1 second = approximately 15 mm. The frequencies for the fifteen intervals were averaged and their probable errors determined.

Fig. 4 is a plot of the variability (*cf.* Crozier, 1929), expressed as per cent probable error of the mean frequency for fifteen tenth-of-a-second intervals, against the number of active sensory groups. The variability is seen to be constant from approximately the seventh sense organ group caudal to the initial incision, while it increases rapidly as one approaches the region of exit of the nerve. The constancy of variability beyond the seventh receptor group indicates that the flattened upper parts of the curve of Fig. 1 and Curve B of Fig. 2 are not due to inability to distinguish and count the higher frequencies.

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pulling on the nerve is inevitable. The subsequent incisions severing the lateral-line canal and nerve at intervals are made with a very thin sharp knife thrust but once vertically into the side from above the lateral-line canal. Spread of damage from these knife thrusts would be slight compared to that from the initial operation where, aside from the effects of mechanical manipulation, diffusion of tissue extracts might produce inhibition of the neuromasts in an amount proportional to their concentrations.

Were this the case one would expect the relative variability to increase with increasing numbers of sense organs owing to the coincidence of impulses on the records which might escape counting.

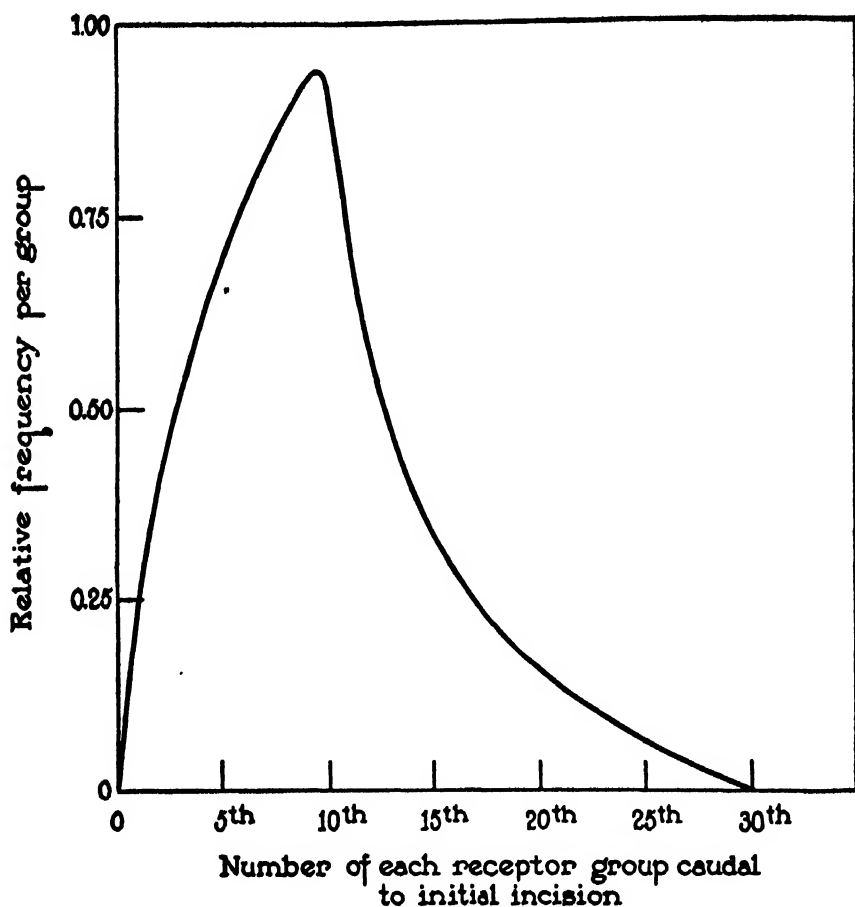


FIG. 3. The differential curve derived from Fig. 1, made by plotting the slope of that curve against the number of receptor groups. The curve shows the relative contribution made by each receptor group to the total response.

Fig. 5 is an example of the data from which the curves have been made. It shows segments of typical records of impulses corresponding to thirty-two, seventeen, seven, four, and two receptor groups.

The frequency and uniformity of the potential magnitudes of the discharge from one or two receptor groups was often so low (cf. Fig. 1,

Curve B of Fig. 2, and Fig. 3) as to indicate that only a few fibers were functioning. It is possible to remove the entire lateral-line nerve and tease apart the branches going to the neuromasts. When this is done one can count under the microscope ten to fifteen fibers going to each group of sense organs, excepting in the closely packed groups near the tail where the number of fibers declines to about half of this number. This paucity of fibers supplying the caudal groups gives a basis for the

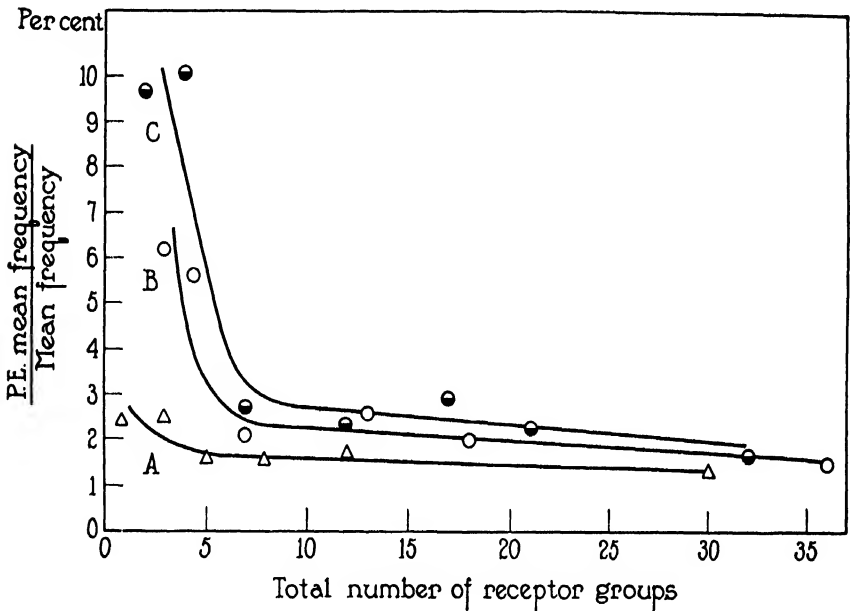
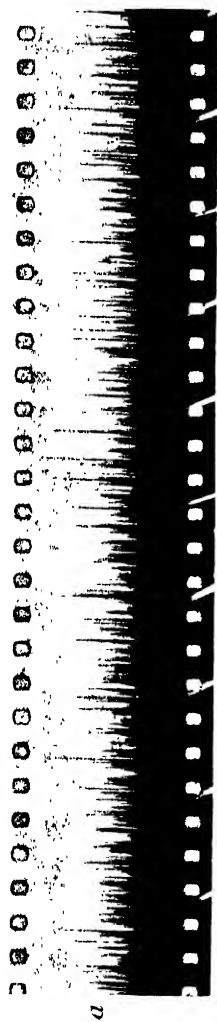
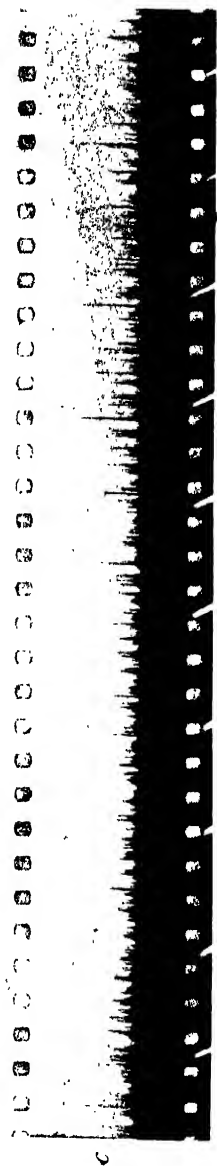
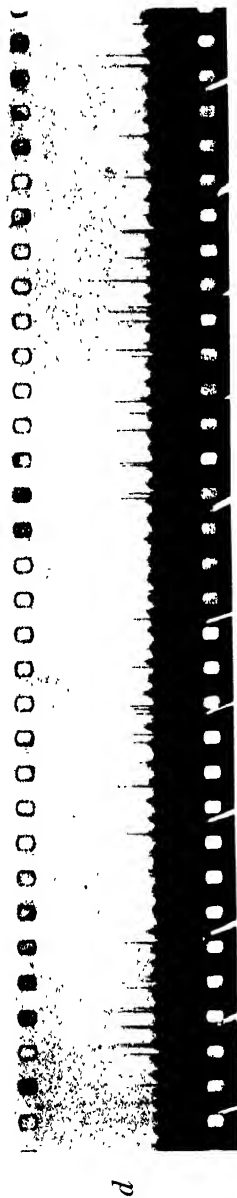
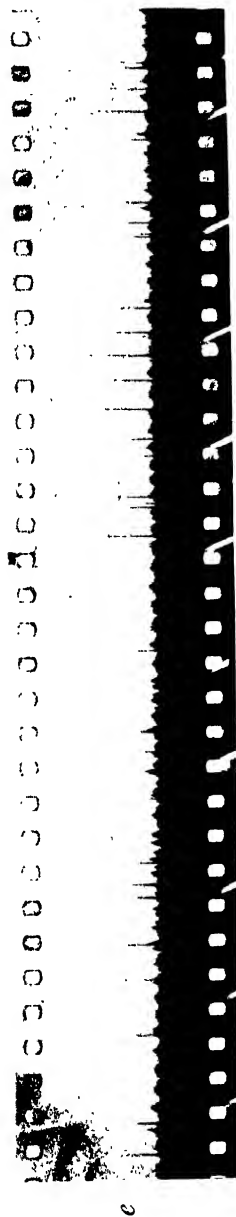


FIG. 4. Plot of relative variation against the total number of receptor groups. The significance of the curves is discussed in the text. Curves A, B, and C correspond respectively to the experiments recorded in Curves A and B of Fig. 2 and Fig. 1.

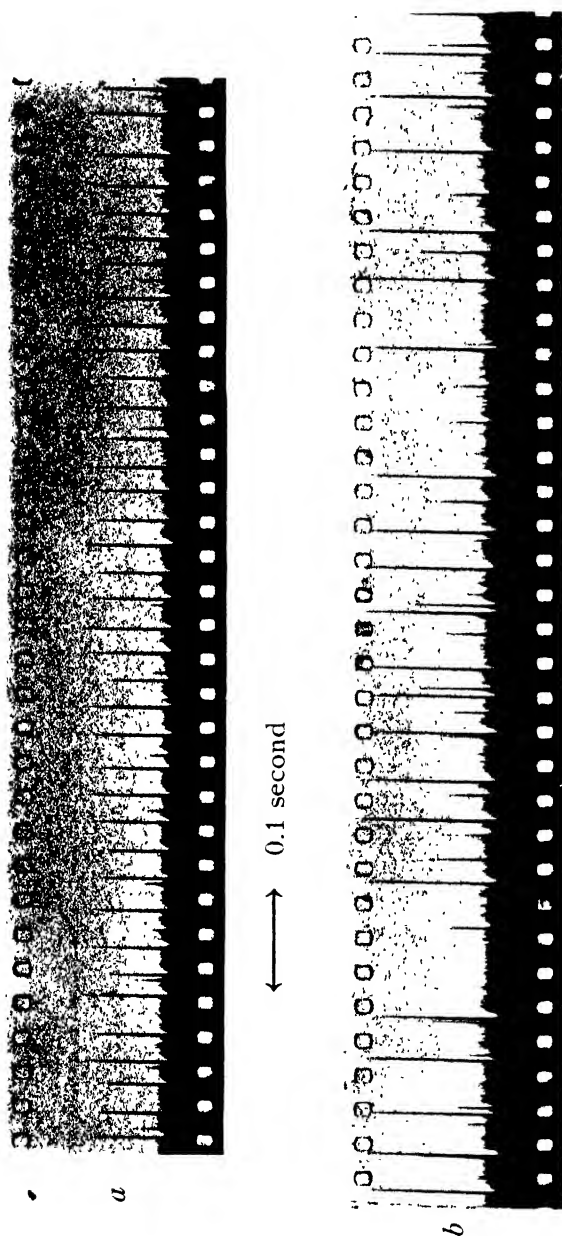
flattened upper parts of the curves of Figs. 1 and 2 and for the declining right half of the curve of Fig. 3. The frequencies are evidently less for the caudal groups because there are fewer fibers supplying them. The fact that the relative number of *active* fibers is few for receptors near the operation compared to those in the middle region of the trunk, while the number of fibers innervating the groups is as large or larger than for those more caudal groups, indicates that some of the fiber connections to the neuromasts near the point of incision are inactive

*32 Receptor Groups**17 Receptor Groups**7 Receptor Groups*

*4 Receptor Groups**d**2 Receptor Groups**e*

←→ 0.1 second

FIG. 5. Sample photographic records of the response for varying numbers of receptor groups. These are samples of the data actually used in determining the curve of Fig. 1. *d* and *e* show responses from only two or three active fibers. Since there are ten to twenty fibers supplying each of the more cephalad receptor groups it is clear that most of these fibers are inactive. Reasons for this inactivity are discussed.



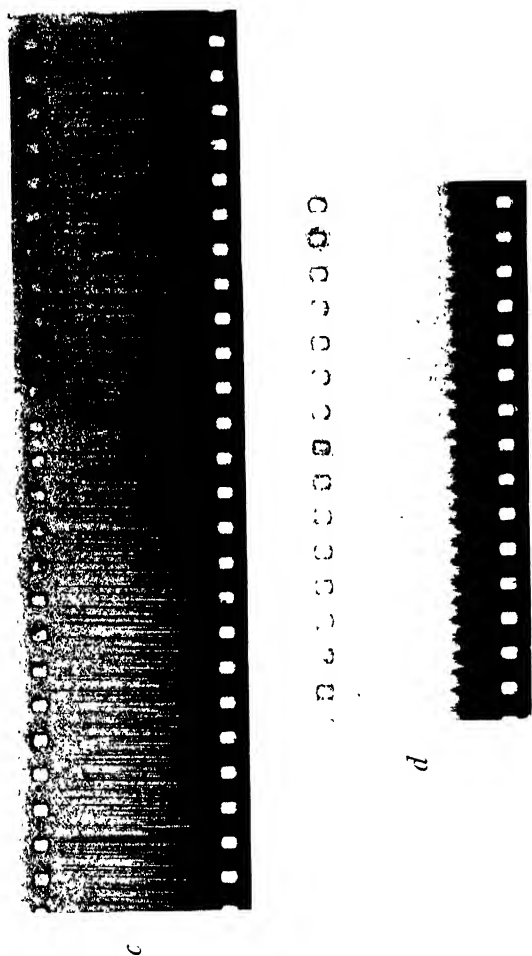


FIG. 6. (a) Rhythmic response of a single fiber obtained by cutting so as to leave only one receptor group and then chilling the skin to 8°C. The photograph was made after 10 minutes at 8°C.  
 (b) Preparation made as in *a*. Temperature 5°C. Two fibers are seen to be active. The waxing and waning of frequency is very characteristic. Cf. also Fig. 5, *d* and *e*.  
 (c) Full spontaneous discharge at room temperature of the lateral-line nerve of a brook trout.  
 (d) Base line immediately after crushing nerve between the electrodes and the body of the fish. Same nerve as in *c*.



(only two or three fibers are active in *d* and *e* of Fig. 5).<sup>2</sup> This susceptibility to damage further strengthens the notion that the inactivity of the lateral-line organs of scaly fishes may be due to destruction of the very delicate nerve fibers characteristic of these animals.

The photographic records at room temperatures of responses from only one or two receptor groups, while indicating a marked decline in frequency as compared to the response from many groups, usually gives a discharge at a frequency too high to enable one to tell the number of active fibers by noting the characteristic ("all or nothing") height of the potential spikes (*d* and *e* of Fig. 5 are exceptions to this). If one chills the skin (*cf.* Hoagland, 1932-33) the frequency declines considerably, due in part to the elimination of certain neuromasts at characteristic temperature thresholds, and it is often possible in this way to get responses below 10°C. which are clearly indicative of the activity of only one or two nerve fibers. The discharge of such single fibers is not perfectly rhythmical but usually waxes and wanes in frequency. A quantitative study of the single fiber response is now in progress. In general, the frequency varies roughly between 40 to 10 per second, the periodicity of the fluctuation from maximum to maximum ranging from 0.5 to 2 seconds. Very rarely a fiber is found which beats quite rhythmically for several minutes without changing its frequency. Fig. 6*a* is an example of this latter response while Fig. 6*b* along with Figs. 5*d* and 5*e* show the waxing and waning type of response found in nine out of eleven cases in which single action potentials were individually distinguishable. Two fibers are shown active in Fig. 6*b*. Fig. 6*c* shows the spontaneous discharge from the lateral-line nerve of a brook trout while 6*d* shows the base line for this same response immediately after crushing the nerve between the electrodes and its exit from the body. The response from the trout is generally more vigorous than that from the catfish.

The falling part of the variability curve shown in Fig. 4 may now be understood, since the increase in the variability evidently depends

<sup>2</sup> The reduced contribution to the total frequency, as indicated by the lower part of the curve of Fig. 1, and Curve B of Fig. 2, and by the differential curve of Fig. 3, is not a characteristic of any particular anatomically fixed group of receptors, but depends only on the proximity of the receptors to the region of the original operation.

upon the fact that the discharge is irregular and fluctuating when few sensory groups are involved at room temperature. This interpretation is borne out by Curve A of Fig. 4 which rises very little when few receptor groups are involved. This curve corresponds to the data of Curve A of Fig. 2, which gives an almost linear rise in frequency for the first five receptor groups. In this experiment enough fibers were active, even from the group nearest the initial incision, to obliterate the uneven discharge due to the individual fibers. Curves B and C of Fig. 4 correspond to the data of Curve B of Fig. 2 and Fig. 1 respectively. The amount of curvature corresponding to the first five receptor groups in Curve B of Fig. 2 and Fig. 1 parallels the corresponding amount of curvature of the variability curves in Fig. 4.

The correspondence of the three variability curves with the three frequency curves serves as an internal check on the analysis. The constancy of variability after the first five or six receptor groups beyond the operation indicates that the method of counting impulses at high frequencies does not introduce an error. The relatively large decline in frequency and the high variability shown in two typical cases, representative of six out of eight experiments, indicates that destructive effects of the operation may extend to the first five or six receptor groups—a distance of roughly  $2\frac{1}{2}$  cm. from the actual operation.

#### IV

If one examines the response of two or three receptor groups as a function of the temperature of the skin, by the method previously described (Hoagland, 1932-33), one gets curves as shown in Fig. 7. The data of two typical experiments out of eleven are plotted. The points are numbered in the order of their determination. It is clear that the decline at low temperatures is not produced by irreversible changes in the tissue, since full recovery occurs on raising the temperature—as is indicated by the points numbered 4 and 5.

Fig. 8 shows data of four experiments plotted according to the Arrhenius equation,  $\frac{K_1}{K_2} = e^{\mu/R (\frac{1}{T_1} - \frac{1}{T_2})}$ , where  $K_1$  and  $K_2$  are frequencies at the absolute temperatures,  $T_1$  and  $T_2$ ,  $R$  is the gas constant, and  $\mu$  is the temperature characteristic. A mean temperature characteristic of 5050 calories is obtained.

Fig. 9 is a plot of the per cent variability against the temperature in degrees Centigrade, for four experiments. Two of the four curves show no change in variability of response with temperature, while the variability is seen to increase at low temperatures in two cases. Since

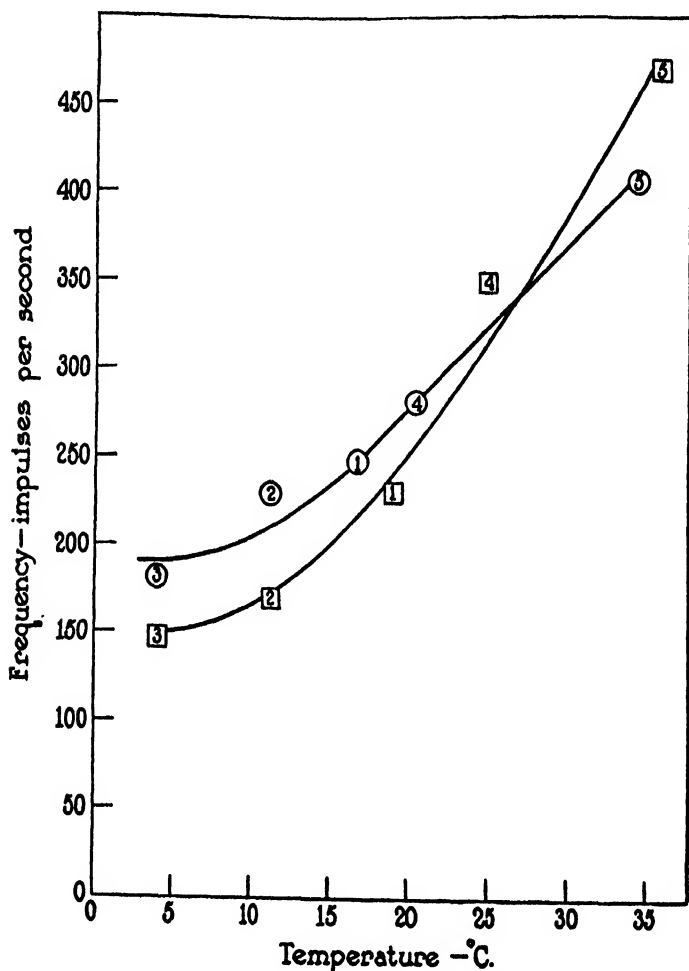


FIG. 7. The numbers of the experimental points indicate the order in which they were determined.

temperature causes a decline in frequency, one would expect the variability to increase at low temperatures if the lowered frequency depended, in part, on the cessation of activity of increasing numbers of receptors with declining temperature. The situation would be similar

to that discussed in connection with the rise in variability of response with the operative removal of functional units illustrated in Figs. 4

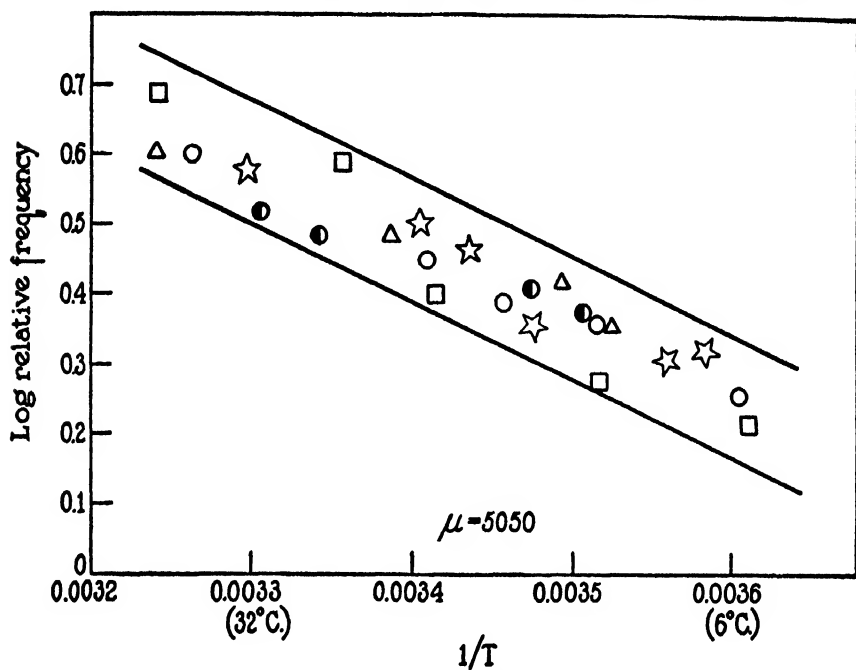


FIG. 8. Plot of temperature vs. frequency according to the Arrhenius equation.

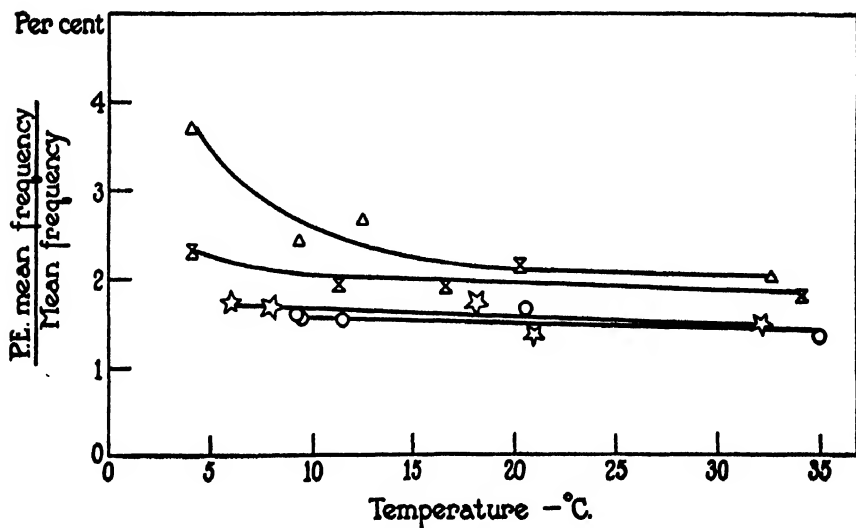


FIG. 9. Relative variability as a function of temperature.

and 5. The rising part of the curves of both Figs. 4 and 9 are evidently the result, therefore, of the elimination of functional units leaving only a few fibers active which, as we have seen, normally beat irregularly. The variation in frequency is, therefore, relatively great. In the experiments illustrated in Fig. 4 the elimination is the result of the effect of the operation on adjacent neuromasts, while in the case of Fig. 9 the elimination is evidently due to the inactivation of neuromasts at low temperature (*cf.* also Hoagland, 1932-33, Fig. 4).

The photographic records of the response also bear this out. For experiments in which the variability is highest one finds that the records indicate that only a few nerve fibers are active at the lowest temperature—the action potential magnitudes can be classified into two or three distinct groups. The variability analysis, therefore, appears to offer an effective approach to the measurement of the number of units which are contributing to the summed response of the nerve trunk. It may also serve as a delicate quantitative indicator of the spread of the effect of surgical manipulation through nervous tissue.

#### DISCUSSION

In attempting to account for the spontaneous discharge from the lateral-line nerve, I suggested (Hoagland, 1932-33) that the hair cells of the neuromasts projecting into the lumen of the lateral-line canal might actually be cilia, which by beating excite the nerve fibers of the neuromasts. As yet it has not been possible to examine the living neuromasts optically. Bowen (1932) has, however, recently reported that the hair cells of the crista of the ear of catfish may be seen to beat as cilia. The sensory region of the crista is made up of sensory cells and supporting cells, the hair cells projecting into the lymph of the ampulla. There is a striking correspondence between the anatomy of the lateral-line receptors and that of the receptors of the inner ear. This close relationship is extended by considerations of the neuroanatomy of the two systems and also by their ontogeny (Herrick, 1901; 1903). According to Bowen the amount and frequency of movement of the hair cells of the crista vary considerably in different preparations. The activity was found to be greatly affected by a variety of reagents, small amounts of which were observed to produce a complete

cessation of movement and a retraction of the hairs. The application of distilled water, for example, caused the complete disappearance of the hairs in from 2 to 3 seconds. In some preparations only one or two hairs were active while in others many were seen to be beating vigorously. Individual hairs were found to beat with waxing and waning frequencies. The average frequency for beating, of four hairs, over a period of some minutes, was 5 beats per second—a frequency somewhat less than that usually found for the spontaneous discharge of fibers of the lateral-line nerve (*cf.* Figs. 5 and 6).

All of the foregoing facts are suggestive if the hair cells of the neuromasts may be considered as cilia. The sensitivity of the hair cells of the crista to reagents suggests a possible parallel with the susceptibility of the neuromasts to operative shock. The character of the beating of individual cilia is to a large degree consistent with what one might expect if similar activity were determining the emission of impulses from the lateral-line nerve.

There is a possible resemblance between the anatomical arrangement of the Purkinje cells of the mammalian cerebellum and the organs of the lateral-line system. In a sense, the latter system may be regarded as a model of a battery of Purkinje cells. In both cases the individual units contribute fibers to a common path. The Purkinje cells are thought to be internuncial neurones which continually receive and transmit impulses serving to regulate posture and coordination by way of tonic excitatory and inhibitory effects on a variety of centers. It has been suggested that they may act tonically by discharging spontaneously. The lateral-line system, in addition to its receptor function, is also known, by its continuous action, to exert a central inhibitory influence (Parker and Van Heusen, 1917, and Hoagland, 1932–33). The quantitative analysis of impulses from the lateral-line system may possibly shed some light on the operation of internuncial neurones.

#### SUMMARY

1. The lateral-line nerves of trout as well as those of catfish are found to discharge impulses spontaneously at a high frequency.
2. The frequency of nerve impulse discharge is measured as a function of the number of participating receptor groups (lateral-line sense

organs). A quantitative analysis is made of the contribution to the total response made by each group of sense organs.

3. An analysis of the variability of the response is presented which makes it possible to estimate quantitatively the longitudinal extent of damage to the neuromasts due to surgical manipulation.

4. A method is described for recording the response of a single nerve fiber in the lateral-line trunk.

5. The frequency of the spontaneous discharge from the lateral-line nerve trunk when plotted as a function of temperature according\* to the Arrhenius equation yields a temperature characteristic of approximately 5000 calories.

6. The variability of the frequency of response as a function of temperature indicates the existence of temperature thresholds for the spontaneous activity of the neuromasts.

7. A possible basis for the spontaneous activity is considered.

It is pointed out that the lateral-line system may serve as a model of the Purkinje cells of the cerebellum.

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# "GROWTH-PROMOTING SUBSTANCE" AND ELONGATION OF ROOTS

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## I

The very marked difference in geotropic response of root and of stem has been explained by implying the existence of different mechanisms, based either on the presence of starch grains in special organs (Nemec, 1900) or on the activity of growth-promoting substances formed by the tip of each organ (Cholodny, 1924).

The first tentative explanation is practically ruled out by the fact that organs of plants which have no starch-containing cells may still show definite geotropic reactions.

The second hypothesis can be considered as favoring one of two possibilities: either different substances are formed by the tips of root and stem respectively, the difference being shown by the reaction of each organ; or else the substances may be the same but elicit reactions of opposite signs by their action on different substrata.

An experiment reported by Cholodny (1924, 1926) gives a possible way of discriminating between the two views under the second hypothesis. He showed that decapitated roots of *Zea* and *Lupinus* when "tipped" again with *Avena* coleoptile tips exhibit geotropic curvature in the right direction and with normal speed, when suitably excited. In other words, substances coming from the tip of a coleoptile determine opposite movements in stem and in root, placing us therefore under the necessity of transferring to the organization of the stem or root the "choice" in the differential response.

If such a substance induces opposite responses in root and stem, one may still wonder if under geotropic excitation of stem or root differential accumulation of this growth-promoting substance takes place (1) at the lower side of the horizontal stem and at the upper side of the horizontal root, or (2) in both organs at the lower side, but with



antagonistic effects; *viz*, accelerating growth of the lower half of the stem and partially inhibiting the growth of the lower half of the root.

Cholodny (1926) has reported that the rate of growth of the roots tipped with coleoptile tips is definitely reduced.

More recently, when the experimental part of the present work was finished, a paper by Keeble, Nelson, and Snow (1931) brought out additional arguments for these views. These authors used *Zea mays* roots, which may not be the very best material for such experiments. Furthermore, the "tipping" was done in all experiments with root, or coleoptile tips and no attempt was made to use the Went-Dolk technique of handling the growth-promoting substance by means of agar blocks.

## II

In the course of an investigation of the mechanism of geotropic bending of roots, the rate of elongation of normal, intact roots of *Lupinus albus* was measured over durations of 2 to 5 hours while the roots were growing vertically downward, at a temperature of 22–22.5°C. Young seedlings with a root of 10 mm. in length on the average were placed on a perforated paraffin disk covering the opening of a small vial, lined almost completely with moist filter paper. Roots were in this way in an atmosphere nearly saturated with moisture. Care must be taken to remove any free drops of water (*cf.* Navez, 1933) which might accumulate at the tip of the root, either by means of a small dry brush or with a little roll of filter paper. The normal elongation was followed by means of a horizontal microscope with micrometric eyepiece. Fig. 1 shows at *A* the type of curve obtained: a straight line fits the observations over the portion of time involved in the experiment. All other conditions being the same, some roots whose normal elongation had been followed for 2 to 3 hours were decapitated at 1.0 mm. to 1.5 mm. from the tip and placed again under observation. The immediate effect of the decapitation is a complete stop in the elongation of the root, followed within 10 to 50 minutes, according to the individual seedling observed, by a renewed elongation proceeding now at a slower rate (Fig. 1, Curve *B*).

For periods of 1½ to 2 hours this new rate remains practically constant, after which it gradually increases. The increase is obviously

due to the regenerated activity of a new "physiological tip" located at the cut section, and is analogous to that observed in the coleoptile of

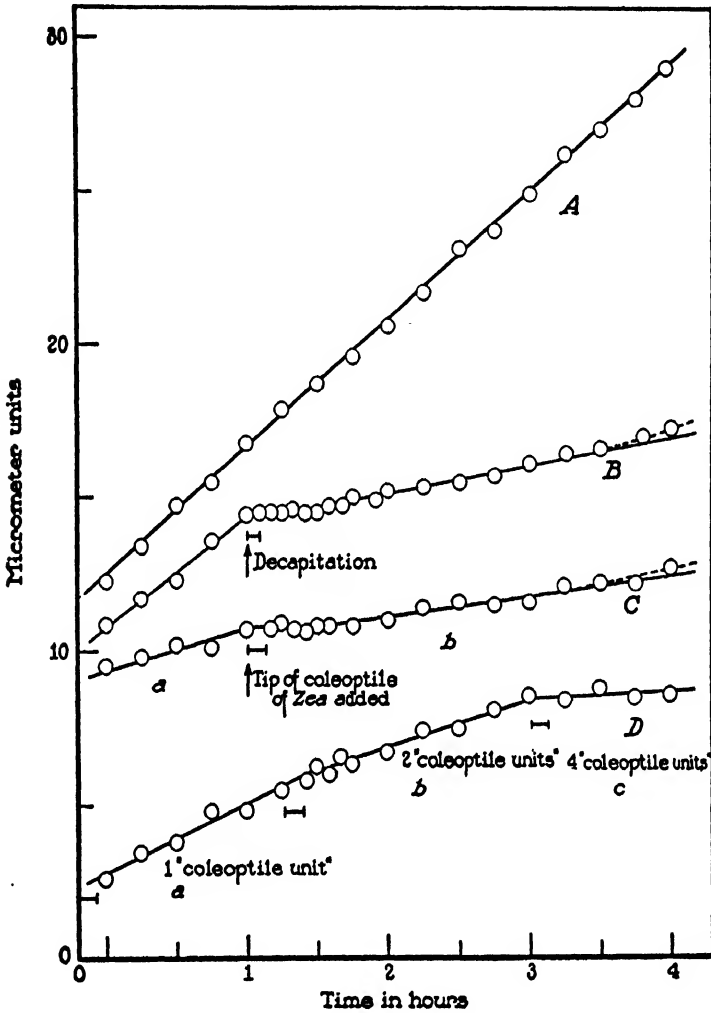


FIG. 1

Curve A. The normal elongation of an intact root of *Lupinus*.

Curve B. Elongation of a root decapitated at the moment pointed by the arrow.

Curve C. A decapitated root elongating at a constant rate (a) is provided at the moment indicated by the arrow with a tip of coleoptile of *Zea*.

Curve D. A decapitated root of *Lupinus* provided successively at the moments indicated by the black lines with 1, 2, and 4 "coleoptile units" of diffusate from *Avena*.

*Avena* by Went (1928). This physiological tip does not involve morphological regeneration, which could not take place in so short a time. The existence of the new physiological tip has been held to be demonstrated by cutting off the last millimeter of the decapitated root, which again reduces the rate of growth practically to what it was before physiological regeneration took place.

Other decapitated roots of seedlings were observed for a period of time sufficient to make sure of a definite constancy in their rate of growth (1 hour); this once established, they were tipped with tips of coleoptiles of *Zea mays* of about 1.5 mm. length, which were made adherent by touching their cut surface with a 3 per cent solution of gelatin. The adhesion of the tips is then very good, and the presence of gelatin does not of itself introduce any factor affecting growth, as control experiments have shown.

One notes in Fig. 1, Curve C, that after a short lag period, which may be attributed to the presence of the gelatin, a constant rate of growth is reached which is maintained for about 2 hours. Apparently the inhibition of elongation then gradually diminishes.<sup>1</sup> The remarkable feature of Part *b* of Curve C is the drop in slope. In other words, the growth substance diffusing from a coleoptile tip reduces the rate of elongation of the root.

### III

To deal in a more quantitative way with the unknown growth substance we used the technique described by Went (1928), and employed *Avena* coleoptiles as the test organism to determine the concentration of active substance extracted by the agar block.<sup>2</sup> Agar

<sup>1</sup> This decrease in inhibition must be attributed to a decreased vitality of the tip of the coleoptile, either through gradual using up of its growth substance or by failure of the tip to receive the necessary materials to keep it in good condition for formation of the effective substance. It is apparently not due to internal conditions in the root, as one can keep the rate depressed by changing at regular intervals of time the tips or blocks of agar containing the diffusate from tips collected by Went's (1928) technique.

<sup>2</sup> Coleoptiles of *Avena* are decapitated when 25 to 30 mm. long, and tipped with agar blocks containing the diffusate of tips. The manipulations are done in the dark room at 22.0–22.5°C., the illumination used coming from a very dim red light of lower spectral limit 635 m $\mu$ . Extractions from tips by diffusion and experiments are done in a chamber the atmosphere of which is nearly saturated in water vapor. Plain agar blocks without diffusate fail to produce any effect on roots. A more complete account of certain aspects of technique will be given in a subsequent paper.

blocks 1 mm. x 1 mm. x 1.2 mm., on which tips of coleoptiles of *Zea* had been placed for 60 minutes, were divided in two halves: one served to determine the quantity of diffusate from the tip, by placing it on a prepared coleoptile of *Avena* and measuring the angle of deflection after 60 minutes; the other half was applied at the tip of a decapitated root. The time curve of elongation of a *Lupinus* root so treated shows perfect parallelism with Curve C of Fig. 1, lending support to the idea that the growth-promoting substance as extracted by this process is really responsible for the observed effect.

Further proof is lent by an experiment involving the use of agar blocks previously in contact with 2, 3, or 4 coleoptile tips. In such a case we get a definite increase of inhibition of elongation when we increase the amount of growth substance provided for the reaction (Fig. 1, Curve D).<sup>3</sup>

#### IV

The inhibition of elongation of a root by stem tip can best be proved if we provide the same root at regular intervals of time with agar blocks containing increasing amounts of growth substance from coleoptile tips. Fig. 1, Curve D, demonstrates this effect.

Another way of demonstrating this point is to place the block of agar in an eccentric position, determining thus an unequal distribution of growth substance in the tissues. In such a case, a definite curvature occurs *towards* the side where the agar block is placed. One remembers that in the case of a decapitated coleoptile the same block of agar induces a curvature towards the opposite side. One can also place symmetrically on either side of a decapitated root two blocks of agar of the same dimensions, separated from one another by a small gap, one of the blocks having been in contact with two or three tips of coleoptile, the other containing only the diffusate of a single tip. In such case, the shape of the root after 3 to 5 hours of contact is definitely

<sup>3</sup> This result is in opposition with the observation of Cholodny (1926) who worked with hollowed-out stems of *Lupinus angustifolium*. The difference between Cholodny's results and ours may be ascribed to the type of contact prevalent in each case: a more or less loose contact between tips and receptive tissue in Cholodny's experiments, a rather perfect contact between agar block and cut section in our experiments. Moreover, the hollowing out of a stem is a more drastic treatment than the removal of the tip.

indicative of the stronger inhibition induced by the agar block containing the diffusate of two or three tips.

Some variation in the experimental results had been noticed which could only be traced to accidental shift in the position of the tips. It led us to investigate the independent reaction in growth of decapitated roots tipped with tips stimulated by gravity. For these experiments the *Zea* coleoptile tips were cut at about 2.5 mm. from the end and were used after definite periods during which they were placed horizontally in Petri dishes with their cut surfaces in contact with agar blocks. Such agar blocks were then divided into two halves corre-

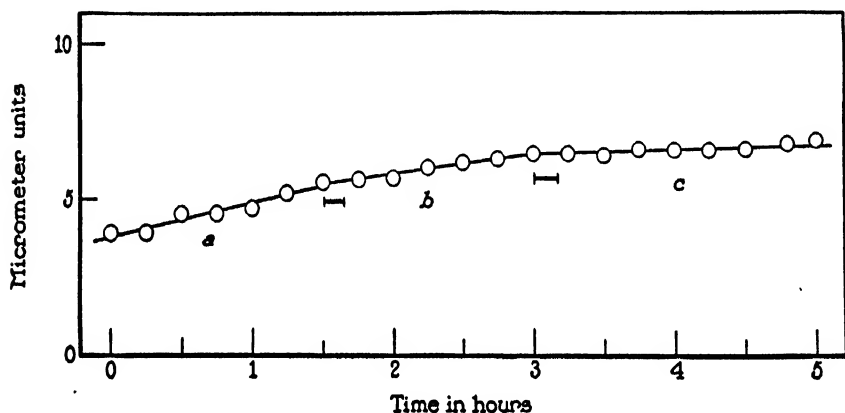


FIG. 2. A decapitated root of *Lupinus* elongating (a) at constant rate is provided (b) with the diffusate of the upper half of a horizontally placed coleoptile tip of *Zea*; in c the diffusate of the lower half is substituted.

sponding to the upper and lower halves of the horizontally placed tips. Each half-block was brought to act successively on the same decapitated root of *Lupinus*. In Fig. 2 the effect obtained by each block is visible: the one corresponding to the lower half has a much greater inhibiting effect on the elongation curve than the upper one.

In the case where each block of agar was placed so as to cover only one half of the cross-section, the deflection of the tip is also definitely more pronounced where the lower half block has been used, corresponding therefore to a more pronounced inhibition of growth on that side.

These experiments support the idea that there is one "growth-

promoting substance" originating in growing tips of root or coleoptile, whose action on decapitated roots results in a lower rate of elongation although the diffusate of the coleoptile accelerates the rate of elongation of the decapitated coleoptile. Experiments have been made with smaller amounts of growth substance from coleoptile tips, as determined by shorter duration of contact of decapitated tip and agar block. The inhibition is very definitely less for 30 minutes' contact; some experiments may even point to a slight acceleration for 10 minutes' contact, although we shall not stress this point before further experimentation is done.

#### SUMMARY

The vertical elongation of normal roots of *Lupinus* seedlings proceeds at constant rate over periods of 4 to 5 hours.

The decapitation of a root stops its elongation for a variable length of time, followed by a period of renewed elongation at a rate lower than that of the normal root.

The tipping of the decapitated root with a tip of a coleoptile of *Zea* induces a decrease in the rate of elongation of the root.

The same effect can be obtained with the diffusate from tips of coleoptile of *Avena* and to a lesser extent with diffusate of root tips.

The reduction in the rate of elongation of the root determined by diffusate from the lower half of the tip of a coleoptile placed horizontally is more pronounced than the inhibition elicited by the diffusate of the upper half of the same tip.

Various experiments with the diffusate of tips support the idea that under the conditions used the growth-promoting substance of the coleoptile tip or root tip inhibits the elongation of the decapitated root.

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## AN ATTEMPT AT PEPTIC SYNTHESIS OF INSULIN

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From the results of numerous experiments published on the chemistry of insulin, it seems that this hormone is a protein having a definite crystalline form (1, 2) and a molecular weight of approximately 35,000 (3). Whether the physiological activity is a property of the whole protein or of one or more constituent groups, has not, as yet, been determined. If it is a property of the whole protein, the possibility of synthesis by chemical means would appear to be somewhat remote. However, there are records in the literature of the synthesis of a protein by other means. Using pepsin, Wasteneys and Borsook (4) have shown that it is possible to synthesise a protein-like substance (plastein) from the products of peptic hydrolysis of egg albumin. Whether their method when applied to peptic digests of insulin would produce a protein-like substance, with or without physiological activity, seemed worthy of investigation. If a plastein was formed during synthesis it seemed reasonable to expect that it might be physiologically active for various reasons: In the first place, insulin, presumably, is synthesised in the body by some enzyme or system of enzymes. Secondly, Beard (5) showed that the plastein obtained in the albumin experiments exhibited a striking resemblance to the original albumin in that the plastein possessed a similar nutritional value. Finally, the possibility of foreign substances taking part in the synthesis of plastein from an insulin digest was greatly reduced by the use of crystalline insulin and crystalline pepsin.

Various workers have reported the results of investigations on the hydrolysis of insulin with pepsin. Dudley (6), and later Witzemann and Livshis (7), showed that pepsin destroys amorphous insulin. Charles and Scott (8), using crystalline insulin, showed that the destruction of the potency of the insulin proceeds at a much greater



rate than does the hydrolysis of the protein. In their experiments they found that when 50 per cent of the protein had been hydrolysed, over 90 per cent of the potency had been destroyed, and concluded that pepsin does not break down crystalline insulin into a simpler, physiologically active substance. More recently, this work has been confirmed by Freudenberg *et al.* (9).

The primary object of the present research has been to study the synthetic action of pepsin in peptic digests of insulin. However, since only small quantities of insulin were available, it was considered advisable to investigate the possibility of repeating the experiments of Wasteneys and Borsook with egg albumin, using quantities of the order of 1 gm. Moreover, it was essential to eliminate any procedure used in their experiments which would inactivate insulin, such as heating at 100°C. Since the synthetic action of crystalline pepsin on the products of peptic hydrolysis of egg albumin has not been reported previously, we are recording our findings in the present paper.

#### EXPERIMENTAL

In general, the procedure used for the hydrolysis and the subsequent synthesis of the protein has been that described by Wasteneys and Borsook. The digestion mixture, consisting of insulin or albumin, in approximately 3 per cent solution together with the pepsin, was adjusted to pH 1.7 with hydrochloric acid. This was incubated at 37°C. for a period varying from 2 hours to 4 days, depending upon the particular experiment. Samples were then removed to determine the extent of hydrolysis, and in the case of insulin, for biological assay. For these tests, it has been our practice to remove five 0.1 cc. samples and dilute them in the following manner. The first sample was diluted with cold isotonic saline, pH 2.5, and the second with cold isotonic saline, pH 8. In order to destroy the enzyme, the third and fourth samples were added to hot (80°C.) acidic and alkaline saline respectively, and after remaining at this temperature for 5 minutes, were cooled to 37°C. The fifth sample was added to 5 cc. of a saturated solution of benzoic acid in ethyl alcohol. About 10 minutes after the addition of this sample, the alcohol was evaporated by heating to 90°C. for about 30 minutes. The insulin was extracted from the residue by shaking with acid water and ether. It has been shown that this procedure will remove insulin from a physiologically inactive insulin-trypsin complex (10). Immediately after the preparation of each sample, it was assayed biologically, using mice (11). The dilutions used were calculated on the supposition that 98 per cent of the potency had been destroyed during hydrolysis. The remainder of the digest was adjusted to pH 4.0 with normal sodium hydroxide and concentrated either by distillation under reduced pressure or by complete removal of the water by sublimation *in vacuo* over concentrated sulfuric acid. When the

latter method was used, the solutions were first frozen by means of solid carbon dioxide. The flask used in the concentration of the digest and the subsequent synthesis was a 200 cc. distilling flask with a small pointed tube graduated at 0.6, 1.25, and 2.5 cc. sealed to the bottom. When the solutions were concentrated by distillation, air was admitted slowly by means of a capillary tube reaching to the bottom of the small tube. This was of assistance in preventing bumping. After concentration, an aqueous solution of pepsin was added and then water to make a final volume of 0.6, 1.25, or 2.5 cc. depending upon the amount of protein used for the experiment. This produced a thick suspension. After the addition of 3 small drops of chloroform as a preservative, the mixture was incubated at 37°C. for about 4 days. At the end of this time, water was added to make the volume the same as it had been before the solution was adjusted to pH 4.0. Most of the suspended material remained undissolved (12). (However, if a sample was centrifuged, the addition of 10 per cent trichloroacetic acid to the clear supernatant to make a final concentration of 3.3 per cent produced a distinct precipitate.) Immediately after dilution, samples were removed from the mixture for the estimation of the total and the non-protein nitrogen, and, in the case of insulin experiments, for five potency tests by the methods already described.

In this research, all determinations of pH were made colorimetrically. It was necessary to use this method since the volume of the digest during synthesis was small. The acidity was always checked immediately before incubation.

The criterion for the synthesis of plastein has been a decrease in the non-protein nitrogen during incubation at pH 4.0. In the samples which were removed to determine the non-protein nitrogen, the protein was precipitated by the addition of sufficient 10 per cent trichloroacetic acid to make a final concentration of 3.3 per cent. Wasteneys and Borsook used a final concentration of 2.0 per cent trichloroacetic acid to precipitate the protein, but it was found, both in the present research and in previous work (8), that this concentration does not ensure complete precipitation of insulin. Consequently, a final concentration of 3.3 per cent trichloroacetic acid has been used in all our experiments. After standing for 1 hour at room temperature, the mixture was centrifuged and the non-protein nitrogen determined in duplicate by the micro Kjeldahl method of Pregl. In the tables the figures listed are for the nitrogen from albumin or from insulin only. The pepsin nitrogen has been subtracted from the non-protein nitrogen fraction, since pepsin is not precipitated under these conditions. In some experiments, the precipitated protein was washed with 3.3 per cent trichloroacetic acid, dissolved in normal sodium hydroxide, and the nitrogen estimated. In all experiments, a record of the samples removed for nitrogen determinations and for biological assay was kept. The nitrogen contained in these samples has been calculated, and the proper corrections made in the tables. In accordance with the method adopted by Wasteneys and Borsook, the per cent synthesis has been expressed as the ratio of the plastein nitrogen to the total nitrogen after synthesis. In a later section of the present paper, it is shown that the value of the non-protein nitrogen after hydrolysis or synthesis can be determined more accurately than

the protein or the total nitrogen. Therefore, the values for the plastein nitrogen have been obtained by subtracting the non-protein nitrogen after incubation at pH 4.0 from the non-protein nitrogen after hydrolysis.

The crystalline pepsin prepared in these Laboratories was obtained from Parke, Davis and Company pepsin (1:10,000) by the method of Northrop (13). Although the product thus formed was crystalline in nature and was much more active than the amorphous pepsin, yet the crystals, when viewed under the microscope, appeared to be mostly spherical in shape and lacked a definite crystalline form. In view of this, it was not certain that the pepsin had been obtained in the most highly purified form. However, on comparing our material with a sample of crystalline pepsin kindly supplied us by Dr. Northrop, it was found that the activity of the two samples was the same. This was done by determining the rate of formation of non-protein nitrogen in a casein solution after digestion with pepsin for 1 hour at 37°C. (14). Using the edestin method of Fuld, the samples of crystalline pepsin were found to possess the same degree of activity, though much higher results were obtained by this method than by the previous one.

In the albumin experiments, Merck's egg albumin was used. The amorphous insulin was prepared in these Laboratories by a method already described (15), and contained 15 units per mg. The crystalline insulin was prepared from this material. It was recrystallised several times from a phosphate buffer solution and had a potency of 25 units per mg.

The exact experimental data for the individual experiments are given below. The object of the first experiment was to determine the possibility of demonstrating synthesis of plastein when a small quantity of albumin was used. Furthermore, it was necessary to modify the procedure of Wasteneys and Borsook in order that it could be applied to experiments with insulin.

*Experiment 1. Albumin-Amorphous Pepsin.*—To 1.00 gm. of egg albumin in 35.0 cc. of water at pH 1.9, were added 75 mg. of amorphous pepsin (Parke, Davis and Company 1:10,000) and 3 drops of chloroform. After standing at 37°C. for 70 hours, samples were removed for the determination of the total, the protein, and the non-protein nitrogen. The rest of the solution was adjusted to pH 4.2 with 1.6 cc. of normal sodium hydroxide and the material concentrated by distillation under reduced pressure to about 2.5 cc. The time of distillation was 2 hours. The temperature during concentration was not above 40°C., most of the material distilling at 37°C. After adding 0.2 cc. of a solution containing 44 mg. of amorphous pepsin and then 3 drops of chloroform, the mixture was set at 37°C. for 69 hours. Water was then added until the volume was the same as that before adjusting to pH 4. Immediately, samples of the mixture were removed and the total and non-protein nitrogen estimated on these. The results of this experiment are shown in Table I.

From this experiment, it is evident that it is possible to demonstrate synthesis of plastein in a digest obtained from a small quantity of albumin. Accordingly, the work was continued, using similar quantities of insulin.

TABLE I

Total nitrogen after hydrolysis, mg. ....	130
Non-protein nitrogen after hydrolysis, mg. ....	124
Hydrolysis, per cent. ....	95
Total nitrogen after synthesis, mg. ....	114
Non-protein nitrogen after synthesis, mg. ....	87
Plastein nitrogen, mg. ....	37
Synthesis, per cent (ratio plastein N/total N) ....	32

*Experiment 2. Amorphous Insulin-Amorphous Pepsin.*—A solution of 1.00 gm. of amorphous insulin in 35.0 cc. of water at pH 1.8 was prepared. To this were added 75 mg. of amorphous pepsin and 4 drops of chloroform. After standing at 37°C. for 50 hours, the solution was removed from the incubator, since hydrolysis seemed almost complete. Five samples were immediately removed for biological assay and diluted in the manner previously described. The tests showed that more than 99 per cent of the potency had been destroyed. Other samples were removed from the digestion mixture and the total and the non-protein nitrogen estimated. The rest of the solution was adjusted to pH 4.0 with normal sodium hydroxide and concentrated under reduced pressure to 2.6 cc. The temperature during distilla-

TABLE II

Total nitrogen after hydrolysis, mg. ....	137
Non-protein nitrogen after hydrolysis, mg. ....	138
Hydrolysis, per cent. ....	100
Total nitrogen after synthesis, mg. ....	110
Non-protein nitrogen after synthesis, mg. ....	82
Plastein nitrogen, mg. ....	56
Synthesis, per cent (ratio plastein N/total N) ....	55

tion was 23°C. 5 drops of amyl alcohol were added to inhibit frothing. The time of concentration was 4 hours. After the addition of 0.2 cc. of a solution containing 44 mg. of amorphous pepsin and 3 drops of chloroform, the mixture was set at 30°C. for 70 hours. Then it was diluted to 35.0 cc. and five samples were removed immediately for biological assay. These solutions were diluted in the usual manner to detect a recovery of 2 per cent of the potency. The tests showed no indication of physiological activity. Other samples of the mixture were removed for nitrogen determinations. The experimental results are indicated in Table II.

Since the insulin-plastein obtained in the above experiment was physiologically inactive, it was thought that this might be due to the fact that hydrolysis had progressed too far. Accordingly, it was decided to attempt to recover the potency using a digest in which hydrolysis had been stopped when the physiological activity had just been destroyed and when only about 50 per cent of the protein had been digested.

*Experiment 3. Amorphous Insulin-Amorphous Pepsin.*—A solution of 1.00 gm. of amorphous insulin in 35.0 cc. of water at pH 1.8 was prepared. To this, 75 mg. of amorphous pepsin were added and the solution incubated at 37°C. for 3 hours. Then samples were removed, diluted, and tested for physiological activity. These tests showed that more than 99 per cent of the potency had been destroyed. After removing 3.0 cc. for the total and the non-protein nitrogen determinations, the rest

TABLE III

Total nitrogen after hydrolysis, mg. . . . .	139
Non-protein nitrogen after hydrolysis, mg. . . . .	72
Hydrolysis, <i>per cent</i> . . . . .	52
Total nitrogen after synthesis, mg. . . . .	139
Non-protein nitrogen after synthesis, mg. . . . .	57
Plastein nitrogen, mg. . . . .	15
Synthesis, <i>per cent</i> (ratio plastein N/total N). . . . .	11

of the solution was adjusted to pH 7.0 with normal sodium hydroxide in order to minimize the possibility of further hydrolysis taking place in the dilute solution during the first stages of concentration. Attempts to concentrate this material by distillation under reduced pressure were not successful due to persistent frothing. The addition of amyl alcohol did not overcome this difficulty. Accordingly, the mixture was frozen with solid carbon dioxide and placed *in vacuo* over concentrated sulfuric acid. At the end of 8 hours, the material in the flask was dry. A small amount of water was added to the powder and the mixture adjusted to pH 4.0. After the addition of 0.2 cc. of a solution containing 44 mg. of amorphous pepsin, the volume was about 3 cc. 2 drops of chloroform were added and the mixture incubated at 37°C. for 110 hours. It was then diluted and five samples were immediately removed and assayed biologically, assuming a recovery of 2 per cent of the original potency. These tests showed no indication of the insulin-plastein possessing physiological activity. Samples of the mixture were also removed for nitrogen determinations. The values obtained in this experiment are given in Table III.

It seemed probable that the failure to obtain any physiological activity in the insulin-plastein synthesised in the above experiments might be due to the presence of foreign substances in the amorphous insulin and amorphous pepsin. The possibility of such impurities taking part in synthesis to form a physiologically inactive plastein, did not seem too remote for consideration. This difficulty could be overcome by the use of crystalline insulin and crystalline pepsin. However, since the synthetic action of crystalline pepsin on a protein digest had not previously been investigated, an experiment with egg albumin was first conducted. This experiment is reported below.

*Experiment 4. Albumin-Crystalline Pepsin.*—To 1.00 gm. of egg albumin in 34.0 cc. of water at pH 1.9, were added 1.0 cc. of a solution containing 39 mg. of crystalline pepsin, and 3 drops of chloroform. After standing at 37°C. for 67

TABLE IV

Total nitrogen after hydrolysis, mg. . . . .	128
Non-protein nitrogen after hydrolysis, mg. . . . .	122
Hydrolysis, per cent. . . . .	95
Total nitrogen after synthesis, mg. . . . .	107
Non-protein nitrogen after synthesis, mg. . . . .	84
Plastein nitrogen, mg. . . . .	38
Synthesis, per cent (ratio plastein N/total N) . . . . .	35

hours, samples were removed for nitrogen determinations and the rest of the solution was adjusted to pH 4. The mixture was frozen with solid carbon dioxide and concentrated by sublimation *in vacuo* (in 4 hours) as in the former experiment. Then 0.35 cc. of a solution containing 13.6 mg. of crystalline pepsin and water sufficient to make a volume of 2.5 cc. were added and the mixture incubated at 37°C. for 113 hours. The mixture was then diluted and samples were removed for nitrogen determinations. The results of this experiment are given in Table IV.

From the above experiment, it is evident that crystalline pepsin can be used to synthesise plastein from hydrolysed egg albumin. Accordingly, it was decided to carry out an experiment using crystalline insulin and crystalline pepsin.

*Experiment 5. Crystalline Insulin-Crystalline Pepsin.*—To 250 mg. of crystalline insulin in 8.6 cc. of water at pH 1.9 was added 0.13 cc. of a solution containing 5.1 mg. of crystalline pepsin. This solution was incubated at 37°C. for 2 hours. Five

samples were then removed for biological assay. These tests showed that more than 99 per cent of the potency had been destroyed. Other samples were removed to determine the total and non-protein nitrogen. The remaining digest was adjusted to pH 4.0, frozen with solid carbon dioxide, and set *in vacuo* over concentrated sulfuric acid for 19 hours. To the dry powder was added 0.075 cc. of a solution containing 2.9 mg. of crystalline pepsin and the volume was made up to 0.6 cc. The mixture was incubated at 37°C. for 83 hours. Then 8.0 cc. of water were added and five samples were immediately removed for biological assay. These samples were diluted to detect a recovery of 1 per cent of the original potency. The tests showed that the material was physiologically inactive. Samples were also removed to determine the total and non-protein nitrogen. The results are listed in Table V.

Several attempts were made to crystallise the insulin-plastein, using methods which were suitable for crystallising insulin (2, 16). No crystals were formed in these experiments.

TABLE V

Total nitrogen after hydrolysis, mg. . . . .	35
Non-protein nitrogen after hydrolysis, mg. . . . .	18
Hydrolysis, <i>per cent</i> . . . . .	51
Total nitrogen after synthesis, mg. . . . .	32
Non-protein nitrogen after synthesis, mg. . . . .	14
Plastein nitrogen, mg. . . . .	4
Synthesis, <i>per cent</i> (ratio plastein N/total N) . . . . .	12

To confirm the results shown in Tables IV and V the experiments with albumin and with crystalline insulin were repeated, using crystalline pepsin prepared by Northrop.

*Experiment 6. Albumin-Crystalline Pepsin (Northrop).*—To 0.500 gm. of egg albumin in 16.7 cc. of water, at pH 1.7, were added 0.8 cc. of a solution containing 13 mg. of crystalline pepsin prepared by Northrop, and 3 drops of chloroform. After incubation at 37°C. for 47 hours, samples of the digest were removed to determine the total and the non-protein nitrogen. The remainder of the solution was adjusted to pH 4.2, frozen with solid carbon dioxide, and set *in vacuo* over concentrated sulfuric acid for 16 hours. At the end of this time, the material was dry. To this, 0.5 cc. of a solution containing 8.2 mg. of crystalline pepsin (Northrop) was added. The volume was made up to 1.25 cc. with water and 3 small drops of chloroform added. After incubation at 37°C. for 72 hours, the mixture was diluted and samples were immediately removed for the determination of the total and the non-protein nitrogen. The results of this experiment are shown in Table VI.

*Experiment 7. Crystalline Insulin-Crystalline Pepsin (Northrop).*—To 0.500 gm. of crystalline insulin dissolved in 16.7 cc. of water at pH 1.8 was added 0.8 cc. of a solution containing 13 mg. of crystalline pepsin (Northrop). After incubation at 37°C. for 2 hours, five 0.1 cc. samples were removed for biological assay. These tests showed that more than 99 per cent of the activity had been destroyed. Other samples were removed to determine the total and the non-protein nitrogen. The rest of the solution was adjusted to pH 4.0 with normal sodium hydroxide, frozen in solid carbon dioxide, and set *in vacuo* over concentrated sulfuric acid for 16 hours. Then 0.5 cc. of a solution containing 8.2 mg. of crystalline pepsin (Northrop) and sufficient water to make a final volume of 1.7 cc. were added. After the addition of 2 small drops of chloroform, the mixture was set at 37°C. for 99 hours.

TABLE VI

Total nitrogen after hydrolysis, mg. . . . .	62
Non-protein nitrogen after hydrolysis, mg. . . . .	64
Hydrolysis, <i>per cent</i> . . . . .	100
Total nitrogen after synthesis, mg. . . . .	58
Non-protein nitrogen after synthesis, mg. . . . .	41
Plastein nitrogen, mg. . . . .	23
Synthesis, <i>per cent</i> (ratio plastein N/total N). . . . .	40

TABLE VII

Total nitrogen after hydrolysis, mg. . . . .	70
Non-protein nitrogen after hydrolysis, mg. . . . .	33
Hydrolysis, <i>per cent</i> . . . . .	46
Total nitrogen after synthesis, mg. . . . .	72
Non-protein nitrogen after synthesis, mg. . . . .	25
Plastein nitrogen, mg. . . . .	8
Synthesis, <i>per cent</i> (ratio plastein N/total N). . . . .	11

Then it was diluted and samples were immediately removed for nitrogen determinations and for biological assay. The latter samples were further diluted as previously described and when tested to detect a recovery of 1 per cent of the original potency, showed no evidence of physiological activity. The nitrogen values are shown in Table VII. Further attempts were made in this experiment to crystallise the synthesised material, but no crystals were formed.

It was considered advisable to investigate the effect on the potency of insulin of certain conditions used in the previous experiments during



the concentration and synthesis of the digest. If these conditions destroyed the activity of insulin, it did not seem reasonable to expect that the insulin-plastein would be physiologically active. These experiments are reported below.

*Effect of Extreme Cold on the Activity of Insulin.*—Since sublimation of solutions frozen with solid carbon dioxide offered an excellent means of concentrating the protein digests preparatory to synthesis, it seemed advisable to investigate the effect of extreme cold on the potency of insulin. Two solutions were prepared: (a) 15 mg. of amorphous insulin dissolved in 5 cc. of water at pH 1.9; (b) 15 mg. of amorphous insulin dissolved in 4.0 cc. of denatured ethyl alcohol acidified with 1.0 cc. of 0.07 normal hydrochloric acid. These solutions were alternately frozen in solid carbon dioxide and melted. This process was repeated nine times, the total time in the carbon dioxide being 12 hours. The solutions were then suitably diluted and biologically assayed. These tests showed that no potency had been destroyed by this treatment.

*Effect of Pepsin on Insulin at pH 4.0.*—It is well known that the potency of insulin is destroyed by pepsin at pH 1.8, but the effect of the enzyme on the physiological activity at pH 4.0, that used during synthesis in these experiments, was problematic. Accordingly, experiments were conducted with insulin and pepsin at pH 4.0 at dilutions comparable with those used during hydrolysis and synthesis. These experiments were carried out in the following manner.

50 mg. of amorphous insulin were mixed with 3.7 mg. of amorphous pepsin in 0.2 cc. of N/10,000 hydrochloric acid. After incubation at 37°C. for 50 hours, 25 cc. of water were added. The acidity was then pH 5.0. The mixture was diluted with isotonic saline, pH 8.0. A biological assay showed that there had been no appreciable loss of potency during the experiment. The work was repeated and the above results were confirmed.

The action of pepsin on insulin in more dilute solutions at pH 4.0 was next investigated. To 10 mg. of insulin dissolved in acid water at pH 2.5, 0.5 mg. of pepsin was added. The solution was then immediately adjusted to pH 4.2. The final volume was 4.7 cc. After incubation at 37°C. for 4 days, the solution was suitably diluted with isotonic saline, pH 8.0, and assayed biologically. These tests showed that not more than 10 per cent of the activity had been lost during the experiment.

Previous investigators have shown that when a suitable amount of trypsin is added to insulin, a physiologically inactive insulin-trypsin complex is formed immediately (17). Since a similar adsorption phenomenon might occur in the present insulin-pepsin experiments, this possibility was investigated. Accordingly, 20 mg. of insulin were dissolved in 0.2 cc. of acid water at pH 2.0. To this were added 1.5 mg. of pepsin in 0.5 cc. of acid water, at pH 2.0. The solution was shaken and immediately suitably diluted with isotonic saline, pH 8.0. A biological assay showed no loss of potency.

## DISCUSSION

From the results of Experiment 1, it is evident that synthesis of plastein from a peptic digest of albumin can be demonstrated with quantities of materials sufficiently small to permit a similar investigation of insulin. Further, the procedure employed was suitable for similar experiments with insulin. The synthesis of the albumin-plastein was obtained by means of amorphous pepsin. In Experiments 4 and 6, an attempt was made to synthesise plastein from albumin digests by means of crystalline pepsin. From the results of these investigations, as shown in Tables IV and VI, it is evident that crystalline pepsin synthesises plastein from albumin digests equally as well as amorphous pepsin.

In Experiment 2, the synthetic action of amorphous pepsin on a peptic digest of amorphous insulin, was investigated. The results of this experiment are tabulated in Table II. They show that 55 per cent of the protein digest was converted into insulin-plastein. Five different samples of this synthesised material were prepared and tested for physiological activity. No potency, however, was detected in any of these tests. It was thought that the failure to recover any physiological activity might be due to the fact that digestion in this experiment had gone to completion, and that the possibility of recovering the activity would be greater if the hydrolysis was stopped when the potency had just disappeared. From previous experiments, it was known that this occurred when approximately 50 per cent of the protein was hydrolysed. Accordingly, in Experiment 3, amorphous insulin was digested until 52 per cent of the protein had been hydrolysed. A biological assay at this time, showed that more than 99 per cent of the activity had been destroyed. A small amount of insulin-plastein was synthesised from this digest, as is shown in Table III. This material, however, when suitably diluted, showed no physiological activity. Since great difficulty was encountered during concentration by distillation under reduced pressure due to persistent frothing, it was found necessary to concentrate the material by other means. This was done by freezing the mixture with solid carbon dioxide and standing it *in vacuo* over concentrated sulfuric acid for a few hours until a dry powder was obtained. Since this method was also used in Experiments 4 and 6 with egg albumin, it is evident that

it does not inhibit synthesis. It was also shown, in other experiments, that the extreme cold used to freeze the mixture does not destroy the physiological activity of insulin since solutions of insulin were alternately frozen with solid carbon dioxide and melted nine times without loss of potency. The success of this method of concentration justified its use in all subsequent work.

In view of the fact that Wasteneys and Borsook (18) found that all fractions of the hydrolysed digest contributed to the formation of plastein, it seemed probable that the impurities present in the amorphous insulin and the amorphous pepsin might have been responsible for the insulin-plastein in Experiments 2 and 3 being physiologically inactive. Since both insulin and pepsin are now available in crystalline form, it was decided to repeat the work using these substances and thus avoid this objection to the former insulin experiments. These experiments, Nos. 5 and 7, have been described, and the results obtained are shown in Tables V and VII. In both experiments, insulin-plastein was synthesised. This material, however, was physiologically inactive.

In the present investigation little work has been done on the insulin-plastein; however, certain observations were made. The plastein was soluble in dilute acid and dilute alkali. It had an isoelectric point at approximately pH 5.0, and in this respect was similar to amorphous insulin. Attempts were made to crystallise the insulin-plastein obtained from digests of crystalline insulin but no crystals were formed by methods which were suitable for crystallising insulin.

In certain experiments, the tables show a decrease in the total nitrogen after synthesis. This is particularly evident in experiments in which concentration was effected by distillation under reduced pressure. This shortage in nitrogen cannot be attributed to removal of material in the distillate, since the nitrogen there was estimated and found to be negligible. Owing to frothing and bumping during the later stages of concentration, considerable material was deposited on the inside of the flask. In certain experiments, it was dissolved in normal sodium hydroxide and the nitrogen estimated. It was found to contain approximately the correct amount of nitrogen to account for the shortage. This material is, presumably, non-hydrolysed protein or synthesised plastein since subsequent dilution and washing with water failed to remove it. For this reason, the value for the

plastein nitrogen in the previous experiments has been obtained by subtracting the non-protein nitrogen after incubation at pH 4.0 from the non-protein nitrogen after hydrolysis. All nitrogen determinations have been made in duplicate. Most of the values agreed within 1 per cent and none of them differed by more than 2 per cent. In some cases, duplicate samples of the digest were removed and a similar degree of accuracy was obtained.

The extent of synthesis obtained in the various experiments is summarised in Table VIII. The values given were determined, as in the experiments of Wasteneys and Borsook, from the ratio of the plastein nitrogen to the total nitrogen after synthesis. However, since hydrolysis in the different experiments was carried to widely

TABLE VIII

	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Hydrolysis.....	95	100	52	95	51	100	46
Plastein N							
Total N after synthesis	32	55	11	35	12	40	11
Plastein N							
*N.P.N. before synthesis	30	40	21	31	22	38	24

\*Non-protein nitrogen.

different degrees, as is apparent from Table VIII, and since the amount of plastein synthesised is partly dependent upon the non-protein nitrogen present, this method of estimation does not afford a true comparison of the insulin experiments with those of albumin. Therefore, it was thought that a better expression of the degree of synthesis would be obtained if the results were expressed as the ratio of the plastein nitrogen to the non-protein nitrogen before synthesis. This also avoids the use of the value determined for the total nitrogen, which, as has already been shown, is subject to a percentage error in certain experiments. The results calculated in this manner are also given in Table VIII.

From Table VIII it is evident that the degree of synthesis obtained in the experiments with egg albumin is of the same order as that obtained by Wasteneys and Borsook. In Experiment.2, in which the amorphous insulin was completely hydrolysed, a slightly greater

amount of plastein was obtained than in the albumin experiments. In other insulin experiments (Nos. 3, 5, and 7) a much smaller percentage of synthesis took place. This was to be expected since only about 50 per cent of the insulin protein had been hydrolysed.

The failure to obtain any physiological activity with the synthesised plastein in any of the insulin experiments, cannot be attributed to the small amount of plastein formed in certain experiments, since the dilutions used in the biological assay were always such that any significant increase in physiological activity during synthesis could be detected. Nor can it be ascribed to an inactive insulin-pepsin adsorption complex, since it was possible to mix insulin and pepsin in the same ratio as in the above experiments, and then recover the activity of the insulin quantitatively. Further, it could not have been due to any secondary reaction of the pepsin on the insulin-plastein at pH 4.0, since it was shown that insulin could be incubated for 4 days with pepsin at pH 4.0 without any appreciable loss of activity.

Since we were primarily concerned in the present investigation with attempts to obtain a synthesised physiologically active plastein from the products of a peptic digest of insulin, little work was done on the products of hydrolysis or on the undigested fraction of the protein. Such investigations might shed much information on the chemistry of insulin.

Although insulin-plastein may bear a similarity to insulin protein, in respect to complexity, still, there must be some difference between these two proteins, since the former is physiologically inactive and cannot be crystallised by methods used for the crystallisation of insulin. The present work with pepsin suggests a similar investigation using trypsin. Although the conditions required to demonstrate synthesis with this enzyme are not so clearly defined, yet, in view of the close association of insulin and trypsin in the pancreas, more interesting results might be anticipated.

#### SUMMARY

1. Synthesis of plastein from the products of peptic hydrolysis of small quantities of egg albumin can be demonstrated with amorphous or crystalline pepsin.
2. Synthesis of plastein from the products of peptic hydrolysis of amorphous or crystalline insulin can be demonstrated with amorphous or crystalline pepsin.

3. The plastein synthesised by pepsin from the products of peptic hydrolysis of insulin is physiologically inactive.

4. The plastein formed in the insulin experiments could not be crystallised by the methods used for the crystallisation of insulin.

5. The physiological activity of insulin is not destroyed by repeated freezing (at about  $-50^{\circ}\text{C}.$ ) and melting of an aqueous or an alcoholic solution of this hormone.

6. No marked decrease in the physiological activity of insulin after incubation at  $37^{\circ}\text{C}.$  with pepsin at pH 4.0, in dilute or concentrated solutions, was detected.

We wish to express our indebtedness to Professor H. Wasteneys for his interest in the work, and to Dr. John Northrop for a sample of crystalline pepsin.

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## THERMOSTAT FOR LOWER TEMPERATURES

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Several years ago we gave a very brief description of a thermostated water bath suitable for experimental work at temperatures below that of the ordinary room as well as above 20–25°C. It seems now desirable to provide a more detailed account of the construction of such units, including the results of experience since 1926. With attention to minor details of construction it is now possible to obtain, at temperatures down to 0°C., a precision of  $\pm 0.001^\circ\text{C}$ . Foote and Akerlof (1931) have described a somewhat similar unit, with best regulation to within  $0.01^\circ\text{C}$ .

The requirements to be met by efficient lower-temperature thermostats for our purposes are rather different from those necessitated by many ordinary problems in physical chemistry. It is required to maintain a temperature below that of the room for sometimes considerable intervals—several days or weeks; in other cases it may be necessary to alter the temperature to a new predetermined level, which will be held precisely, at intervals of an hour or so. Simplicity of construction is essential, with interchangeability of parts, when a number of installations are in use. The degree of precision attainable should be considerable. It is not infrequently supposed that since biological systems are intrinsically variable in their performance, quantitative measurements are significant only to a degree of exactness permitted by the statistical character of the intrinsic, organic variation. This conception is entirely erroneous. With utmost possible care directed to the reduction of variance due directly to external influences and to fluctuations in such influences, for example in the temperature, it becomes possible to investigate the intrinsic variance of performance as a property of a given biological system; this is done by considering the variance as a function of precisely adjustable magnitudes of independent



variables (Crozier, 1929). It is impossible to conduct analytical dissection of fluctuations in performance without the most rigidly attainable control of the extra-organic variables involved in a given experiment.

### *Cooling Unit*

For water baths of small volume, an immersible funnel-type cooling unit packed with cracked ice has proved handy, efficient, and quite accurate (Stier, 1931).

Standard equipment, which may be used for short time or for long period operation, is based upon the use of an SO<sub>2</sub> compressor, or of a compressor using some analogous substance. The compressor used is usually of the piston type. 30-foot coils of  $\frac{1}{2}$  inch copper tubing are fashioned for each thermostatic bath; these coils are commonly 7-8 inches outside diameter, and the turns are spaced  $\frac{1}{2}$  inch apart to permit good circulation of water. The expansion valve above water is attached by a length of  $\frac{1}{2}$  inch copper tubing to the first turn, at the bottom of the coil. (Rotary type compressors, factory-assembled and sealed, with the commercially prepared coils permanently attached, have been used for certain installations; in this case the shelves for making ice cubes are removed.) These units are used for larger baths. The largest we have used with success contains 55 gallons.

When air incubators are to be used at temperatures below that of the room they are most efficiently placed in cold rooms whose temperature is set about 5° below the temperature wanted in the incubator. We have also used small commercial electrical refrigerators, replacing the thermostat with our own thermoregulator and relays. The cost is less than that of a laboratory incubator of similar size. The precision of regulation is about  $\pm 0.3^{\circ}\text{C.}$ , even without a circulating fan.

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FIG. 1. Thermoregulator tube and carriage for suspension. One side of the U-tube is closed by a stop-cock (5). It communicates with a suction tube (1), with a cotton plug as air filter. The lead (4) makes contact through a sealed-in platinum wire (6). The connecting wire (2) is held by a rubber cap (3). The other side of the U-tube, bearing the capillary, is connected with the two other limbs by a solid glass cross rod for support. The needle (18) for contact in the capillary is carried on a fine screw (15) provided with a lock nut (16); the screw works in a brass collar (17) which grips the upright tightly; it carries a binding post for the lead wire (2).

The mercury-filled tube is carried in a brass stirrup (9) with a cross member (7); the stirrup is of  $\frac{3}{4}$  inch angle-brass; where it makes contact with the glass, sponge rubber buffers are inserted (8, 10). At its upper end the stirrup is borne by a cross member to which the stirrup is bolted; the threaded ends of the bolt are fitted with cotter pins. The cross member is supported by heavy rubber bands (13) passing over rollers (12). There is a second cross piece (11) with a central hole for attachment to a stand. In case of breaking one or both rubber bands, through long use, a chain (14) prevents dropping of the thermoregulator to the bottom of the tank.



### *Heating Units*

Three convenient methods are available for adding heat to the thermostatic tank: (1) current may be sent through the copper coils of the cooling unit (*cf. e.g.*, Schmitt and Schmitt, 1932), employing a transformer; (2) show-case lamps, painted black, and ordinarily of 40 watts; (3) knife-type heaters, of 50 watts, for relay operation, or of 125 watts if used for higher temperatures.

### *Thermoregulator*

When it is desired to change the temperature at frequent intervals, as often as once every hour, an open-end U-type thermoregulator is used, filled with redistilled mercury. The capacity of the standard regulator is 3.7 pounds of mercury. The capillary in which contact is made is 0.025 inch bore. A stop-cock at one end of the U, with a slight enlargement above it, permits easy changing of the volume of mercury in the regulator proper. A diagram of this regulator, which includes ideas derived from a number of sources, is given in Fig. 1. Chattering at the relays is largely eliminated by a special spring suspension supporting the regulator. This is quite important when the regulator is used to start a  $\frac{1}{4}$  h.p. motor in the compressor unit, since the starting load is heavy. Contact with the mercury is made by a No. 28 platinum wire sharpened to a point. Tungsten is also satisfactory, but the "sensitivity" of constantan seems to vary a good deal.

When it is desired to maintain constant temperatures for long periods, weeks or months, single tube thermoregulators filled at the top with inert gas and completely sealed are preferable. The difficulty of precisely setting these regulators prohibits rapid work with them.

### *Stirring Motor*

A satisfactory stirring motor for use in a thermoregulated bath must have the following characteristics: (1) variable speed, adjusted by rheostat or step transformers; (2) no "radio interference;" (3) it should have grease-packed ball bearings, reducing servicing; (4) it should be silent in operation, since the observer's head must frequently be close to it; (5) it must be capable of running continuously for at least a year; (6) low cost.

These specifications are effectively met by a condenser type motor of 1/20 to 1/15 h.p. Both end bells are ventilated, and Bakelite insulation is provided on the wiring in the motor. The shaft of the motor extends 2 inches beyond the motor housing. A brass coupling with lock screws unites the shaft to a bronze propellor  $1\frac{1}{4}$  inches in diameter. The propellor blades throw water downward, but the rotation can be reversed. The housing of the motor is arranged for vertical or horizontal mounting. The lower end bell has 3 lugs cast upon it, so that the motor may be hung from a wall bracket, or from the ceiling. Or the motor may be mounted upon a horizontal support resting on "live" rubber. A "C" clamp sup-

port may be attached to the side of the motor through bolt holes provided in the motor casing, and permits attachment to the side of the tank or to a table near the tank.

Motor-driven propellers do not always give the most effective stirring for some purposes. To produce a uniform top-to-bottom movement of the liquid in the bath, water may be drawn from the top by a centrifugal pump which returns the water to a horizontal brass pipe, on the floor of the tank, with apertures along its length. This method of agitation is desirable for long thermostat tanks, particularly if they carry a projecting shoulder at one side.

### *Relays*

The weakest link in any system of thermostatic control of tanks is always to be found in the relays. We have persistently endeavored to keep the relay device as uncomplicated as possible. The general purpose and aim have been to reduce the operating current of the relay to the smallest possible, thus reducing oxidation at the mercury surface of the regulator. We have used many more complicated schemes than the one here to be outlined, including amplification devices (Beaver and Beaver, 1923; Roberts, 1925; and others), and Thyatron valves (*cf.* Schmitt and Schmitt, 1932).

We have used with good success secondary relays of the latch type (operating on 110 volts A.C.) actuated by a primary relay (operating on 1 ma. at 6 volts D.C.) in series with the thermoregulator. The oxidation at the mercury surface in the capillary of the thermoregulator is practically negligible with such a low operating current for the primary relay. Since the latch type of relay gives greater pressure at its contacts and is more positive and powerful in its action, "sticking" of the contacts (often a serious difficulty when a  $\frac{1}{4}$  h.p. motor is to be started with an 8 ampere starting load) is eliminated. Sticking of relay contacts can also be avoided by using gas-filled mercury switches, or vacuum contacts which are actuated by the armature of the relay. Under laboratory conditions where a large number of relays are employed we have found these glass-enclosed switches to be too fragile for general use.

A cheaper relay which is also serviceable is easily made from an ordinary 20 ohm telegraph relay, single pole, double throw type, operating on 6 volts. If used directly, very considerable arcing is apparent at the mercury surface in the capillary of the thermoregulator. This is entirely obviated by connecting an additional coil of 20 ohms resistance in series with the coil of the relay; when the thermoregulator circuit is made, the thermoregulator mercury short-circuits the additional coil. Large silver contacts, 1 inch in diameter, insulated from each other, replace the standard fixed contacts supplied by the manufacturer. The armature carries a solid, double-faced carbon contact. For dependability over long periods of time, it is advisable to use a secondary power relay of the double pole, single throw type operating on 110 volts A.C. for starting the motor of the compressor. This combination can be purchased for about five dollars.

### *Control Cabinet*

A sheet metal box with removable cover and hinged back has been developed as a standard control unit (see Fig. 2). It contains the relay mechanism, the rheostat controlling the speed of the stirring motor, and on the front face a 10 gang ordinary commercial receptacle set carrying permanent connections to the units in the control cabinet. One has then only to plug into the front panel with standard plugs, in order to secure the proper interconnections. In ordinary laboratory operation this is important as avoiding short circuits, as disconnected cords are always "dead." Switches controlling A.C. and D.C. lines which enter the control cabinet are provided with suitable pilot lamps and separate fuses, the latter precaution preventing raids upon wall panels.

### *Tanks*

The thermostated tanks are made of sheet copper throughout. If this is not done, electrolytic corrosion results in connection with the copper coils of the cooling unit. All apparatus dipping into the tank must be of brass, or heavily copper-coated. The wall of the copper tank is double, an outer shell of sheet copper being provided, which is coated with Bakelite or clear lacquer to prevent tarnish. The  $\frac{1}{4}$  inch space between the tank and the outer shell is filled with thin sheets of cork, which give very effective insulation; the sheet form of the cork gives additional air spaces. The edge of the tank is of angle-iron, copper-covered, for attachment of clamps (see Fig. 2).

For purposes of observation plate glass windows ( $\frac{1}{4}$  inch thick) are inserted at various positions on the sides, or in the bottom. Such windows may be made water-tight by one of a number of devices. The most effective consists in mounting the plate glass in a copper frame, using a mixture of white lead with the best grade of varnish; the copper frame is then soldered to the wall of the tank. Emptying tanks is facilitated by 1 inch cocks built into the bottom.

For use with Warburg manometers, and for certain other purposes, a shoulder may be provided on the long side of the thermostated tank; the floor of this projecting shoulder is made of plate glass (Fig. 3).

### *The Operation of the Thermostat to Obtain Precision $\pm 0.001^{\circ}\text{C}$ .*

The best thermoregulation is of course obtained with adjustment of the gas pressure in the compressor so as to give the shortest cycle of operation of the cooling unit. Morgulis and Beber (1927) used an  $\text{SO}_2$  compressor operated continuously in conjunction with heating lamps acting intermittently through a thermoregulator; the precision of regulation was only  $0.1^{\circ}\text{C}$ . We have used the same general method and have obtained a precision as great as  $\pm 0.002^{\circ}\text{C}$ . in the neighborhood of room temperature. The labor of adjustment



FIG. 2. A standardized thermostatic bath, showing mountings of tank, cooling unit, stirring motor, and thermoregulator, as well as control box.

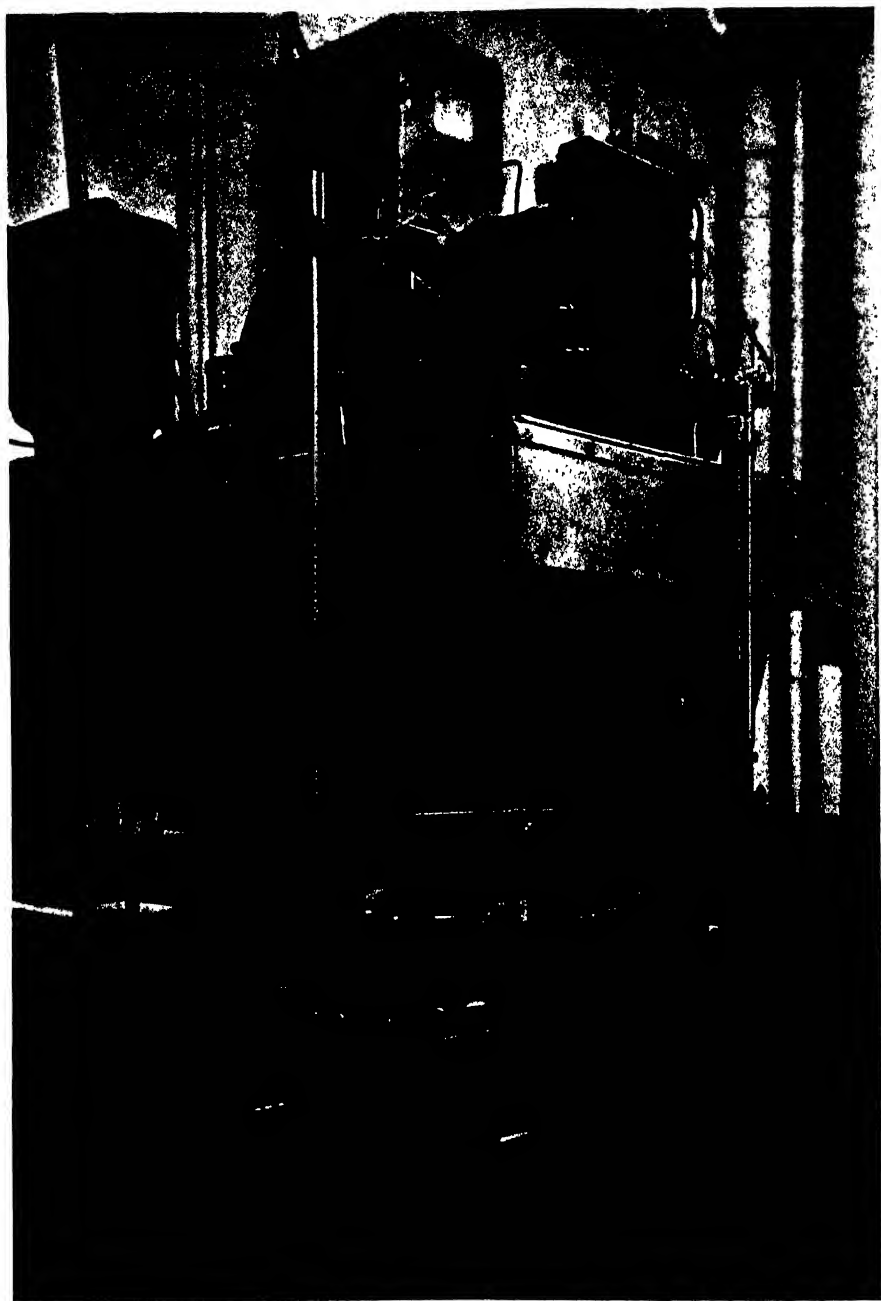


FIG. 3. A standardized thermostatic tank with shoulder at one side, for use with manometer vessels. This particular unit is cooled by a commercial rotary type compressor with the customary coils permanently attached (see text).

of action of the compressor, and of the heating elements, is altogether too great, with this method, particularly when the temperature is to be newly adjusted at frequent intervals.

During one cycle of the cooling unit, with intermittent operation, the following changes are detectable: Immediately after the compressor starts to work, the temperature of the well-stirred bath rises. The rise may be as much as  $0.006^{\circ}\text{C}.$ , and depends upon the setting of the expansion valve in the cooling coil and upon the length of the preceding cycles. During the activity of the compressor the temperature of the bath gradually falls. When the compressor is stopped by the interruption of the thermoregulator switch, the temperature of the bath continues to fall; the magnitude of the fall, and its duration, depend upon the setting of the expansion valve and the working time of the compressor; the excess fall in temperature may be as great as  $0.008^{\circ}\text{C}.$  Part of this fluctuation is due to lag in action of the thermoregulator. Attempt has been made (Fig. 1) to adjust suitably the relations of volume of mercury to surface exposed.

A short cycle of operation in the bath has been attained, with corresponding increase in precision, by three refinements. (1) The lag is reduced by placing the thermoregulator beside the cooling coil. (2) A 50 watt knife heater is located near the thermoregulator, and so connected that the heater is in operation when the cooler is stopped. The relay is of single pole, double throw type, making one circuit when the relay is energized, making the other circuit when the relay is not activated. (3) The setting of the expansion valve on the cooling coil is so adjusted by trial that there is a minimum of additional cooling after the compressor motor has been stopped by the action of the regulator.

Under these conditions a precision of  $\pm 0.001^{\circ}\text{C}.$  is obtainable in a tank holding 20 or 30 gallons of water, even when no cover is used. Tests are made with a Beckman thermometer tapped continuously.

We wish to record our obligation to Mr. E. A. Gibbs and Mr. A. B. Small, of the Engineering force of Harvard University, for their kind assistance in connection with the development of these installations.



## SUMMARY

Details are given concerning the construction and operation of relatively simple thermostats which permit maintaining precise temperatures down to 0°C. (with water), or temperatures above that of the ordinary room, and in which the temperature may be quickly altered at short intervals to new levels.

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# THE DIGESTION AND INACTIVATION OF MALTASE BY TRYPSIN AND THE SPECIFICITY OF MALTASES

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In 1925 Leibowitz (1) proposed the theory that there are two kinds of maltases,—one kind present in yeast, having the power of hydrolyzing maltose and  $\alpha$ -methylglucoside and another found in moulds (2–4) with only maltose-splitting power. Thus he would differentiate between glucosidomaltase and glucomaltase. But Weidenhagen (5) has taken quite a different view. He thinks that sucrose may be hydrolyzed by  $\alpha$ -glucosidase and by a  $\beta$ -*h*-fructosidase, and maltose only by  $\alpha$ -glucosidase. This he explains by his steric configurative theory, according to which one and the same enzyme,  $\alpha$ -glucosidase, must be able to hydrolyze sucrose, maltose, and  $\alpha$ -glucosides since each has an  $\alpha$ -glucosido rest bound to a glycone or an aglycone. In other words this means that the classical nomenclature of maltase, sucrase, etc., would have to be dropped, since such separate enzymes would not exist. Recently we (6) have shown the maltase of the mammary gland to be unmistakably a glucomaltase, hydrolyzing maltose but not sucrose or  $\alpha$ -methylglucoside, thus corroborating the theory of Leibowitz. Doubt has been cast upon Weidenhagen's work (7) from another angle by Karstroem (8), Myrbaeck (9), and Virtanen (10) who found that the enzyme of a certain strain of *B. coli* can hydrolyze maltose but not cane sugar. Experiments on moulds by Pringsheim, Borchard, and Loew (11) speak also against the theory of Weidenhagen.

Since, in enzyme chemistry, the question whether there are distinct maltases and sucrases is of considerable importance, we have undertaken to procure additional data by studying the maltase of saliva and the maltase produced by *Escherichia coli*. For the determination of enzyme activity we have used an improved technic. We have utilized our new method (12) which permits of the determination of minute

amounts of monoses in the presence of bioses. We have also investigated the chemical nature of the maltase of *E. coli*. We shall show that in contrast to the difficulty with which some other enzymes respond to tryptic digestion, this maltase is digested by trypsin with remarkable ease within a relatively short time.

#### EXPERIMENTAL

The organism used was a laboratory strain of *E. coli* (*B. coli communis*), obtained in 1930, which ferments glucose, maltose, lactose, with acid and abundant gas formation. The enzyme (maltase) was prepared<sup>1</sup> according to the directions

TABLE I

*Experiments Showing That the Maltase of E. coli (B. coli communis) and That of Saliva Are Glucomallases*

Temperature 37°

Nature of preparation	Hydrolysis of maltose	Hydrolysis of sucrose	Hydrolysis of $\alpha$ -methyl- glucoside	Time of incubation
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>hrs.</i>
<i>E. coli</i> .....	14	None	None	2
" " .....	40	"	"	20
" " .....	51	"	"	72
Saliva .....	21	"	"	20
" .....	30	"	"	40

of Karstroem (8) with the only difference that we have used 600 ml. of culture material instead of 6 liters. A sterility test was made from the enzyme material. The bacteria-free enzyme preparation was dissolved in 10 ml. of distilled water. 1 ml. of this solution had a dry weight of 6 mg. To 2 ml. of this or to 2 ml. of saliva, 1 ml. of buffer (pH 6.9 N/10 acetate) and 1 ml. of 1.42 per cent maltose, 1.42 per cent sucrose, or 1 per cent  $\alpha$ -methylglucoside, respectively, were added. Toluene was used as an antiseptic in all experiments. At intervals 0.5 ml. samples were taken from the digests and the protein precipitated with 95 per cent ethyl alcohol. In the case of maltose 10 volumes of alcohol and in that of sucrose 5 volumes were used for the precipitation. Boiled samples of the enzymes were run as controls. In 1 ml. of the clear filtrate the degree of hydrolysis was determined by our monose method (12). The results of these experiments are summarized in Table I. It is quite evident that the maltase of both *E. coli* and saliva

<sup>1</sup> For this preparation we are indebted to the Department of Bacteriology of this College.

hydrolyzes maltose fairly easily while having no effect upon sucrose or  $\alpha$ -methylglucoside.

*Inactivation of Maltase by Tryptic Digestion.*—To 2 ml. of the maltase solution of *E. coli*, there were added 2 ml. of 0.3 per cent trypsin (Fairchild Bros. and Foster) dissolved in N/10 acetate buffer of pH 6.9. It was incubated at 37° for 2 days. After this 1 ml. maltose (1.42 per cent) was added and it was again incubated for 2 days. We found that after 2 days of tryptic digestion all the maltase was inactivated. A boiled trypsin buffer solution, and a boiled maltase buffer solution, respectively, served as controls. It should be noted that there is a spontaneous hydrolysis of substrates even in water solutions, which should never be neglected when testing for enzyme activity. Since the 0.3 per cent trypsin solution gave no immediate precipitate with 95 per cent alcohol and the maltase solution gave a heavy precipitate it was easy to follow proteolysis by comparing the precipitates given by the digest and the original maltase solution. After 2 days tryptic digestion the inactive maltase solution gave no precipitate with 95 per cent ethyl alcohol.

#### DISCUSSION

In Table I it is shown that the maltase of saliva does not act in accordance with the theory of Weidenhagen. It only hydrolyzes maltose—not sucrose and not  $\alpha$ -methylglucoside. Nor does the maltase of *E. coli* (*B. coli communis*) hydrolyze anything but maltose, which is in confirmation of the work of Karstroem, of Myrbaeck, and of Virtanen, who have also studied the enzyme-producing power of some of the organisms of this group. Our experiments on the specificity of “saccharases” support the theory of Leibowitz, who claims the existence of two kinds of maltases: glucomaltases, of which the maltase produced by *E. coli*, the maltase of saliva, and the maltase of the mammary gland are examples; and glucosidomaltases, of which yeast-maltase is an example, hydrolyzing both maltose and  $\alpha$ -methylglucoside. The use of the special monose reagent which is very sensitive to monosaccharides but is not changed by disaccharides (12) has given us an extremely definite analytical procedure for testing the point mentioned.

Contrary to the findings of Pringsheim and Leibowitz (13), who think that there is no maltase at all in saliva, it was found in a series of experiments (14) that maltase is a constant constituent of the saliva. This was believed to be the case by earlier workers. It varies greatly in different individuals, some having only a trace of maltase

in their saliva. However, even such a trace is not negligible if it is remembered that the organic dry weight of the saliva is not more than 0.5 per cent.

As regards the digestibility of maltase, it is well known that much of the maltase of yeast is inactivated during the autolysis of the yeast cells, which is a part of the procedure used in preparing yeast-maltase. This has been attributed to the increasing acidity of the autolysate (15). However, in view of our experiments which show that maltase is digested and inactivated by trypsin, it is possible that the inactivation of yeast-maltase during autolysis really may have been due to the action of proteases, which are found in abundance in yeast, and which act best at a pH ranging between 4.0 to 7.8. Some enzymes (16) are not digested by trypsin, and crystalline urease is inactivated by trypsin only if a gum is present (17, 18). Sumner and Kirk (19) could not digest crystalline urease with trypsin, with or without gum, but Sumner, Kirk, and Howell (20) digested crystalline urease with pepsin and with papain. It has been shown by Northrop and Kunitz (21) that crystalline trypsin is digested and inactivated quite rapidly by dilute solutions of crystalline pepsin. It was found by us (18) that concentrated solutions of trypsin may prevent the digestion of crystalline urease, by acting as a protective colloid for the urease. For this reason dilute trypsin solutions were used in these experiments on maltase.

#### SUMMARY

1. The maltase of saliva and that of *E. coli* (*B. coli communis*) hydrolyze maltose but not  $\alpha$ -methylglucoside or sucrose and are therefore to be considered glucomaltases.

2. Maltase is rapidly and completely inactivated and digested by trypsin.

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# ON THE RELATION BETWEEN MEASUREMENTS OF INTENSITY DISCRIMINATION AND OF VISUAL ACUITY IN THE HONEY BEE

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## I

A method for study of *discrimination of photic intensity* by the honey bee, and certain experimental results, have been described (Wolf, 1932-33). It was shown that the bee's discriminating power for different brightnesses varies with illumination in much the same way that this function does for the human eye (Koenig and Brodhun, 1889; Hecht, 1924-25). The discriminating power is poor at low illuminations; as the intensity of illumination increases the discrimination increases, ultimately less rapidly, until it reaches a certain level at high illuminations. The total range over which the bee can distinguish between different intensities was found to be very much smaller than for the human eye, and at an illumination where the discriminating power of the human eye and of the bee's eye are at their best, the intensity discrimination by the bee is one-twentieth as good as that by the human eye. The experiments by which these results were obtained were made under "optimal" conditions as regards the visual acuity of the bee's eye; for test object a pattern of stripes was chosen which could be easily reacted to even at the lowest illuminations used during the experiment.

The *visual acuity* of the human eye varies with illumination in such a way that at low illuminations the resolving power is poor; at higher illuminations it increases, until it finally reaches a maximum level (Koenig, 1897; Hecht, 1927-28). The same kind of relationship was found for the faceted eye of the bee (Hecht and Wolf, 1928-29). While the relation between visual acuity and illumination is the same in principle for the two organisms, there is a tremendous difference in



the absolute magnitudes of the visual discrimination powers. The bee's visual acuity at its best is lower than the lowest human visual acuity. Under maximal conditions for each eye the fineness of the resolving power of the human eye is about one hundred times that of the bee.

No data have been available concerning the relation between visual acuity and intensity discrimination. Accordingly, an investigation of this relationship has been made, with the bee's eye. The procedure was in principle the same as in the previous studies (Hecht and Wolf, 1928-29; Wolf, 1932-33).

## II

### *Apparatus and Procedure*

If the visual field of a bee is made up of a pattern of alternating dark and illuminated bars, or of alternating bars of different brightnesses, the animal will respond to any displacement of this field as long as there is maintained not less than a certain minimum difference in the brightnesses of the alternate bars. In case the animal cannot "resolve" the pattern, either on account of the width of the stripes at a given illumination, or on account of a smaller difference in brightness of alternate bars than is necessary for distinction, the field will act as if uniformly illuminated and a displacement of the field will not elicit a response.

The general method for testing the visual acuity and the intensity discrimination of the bee has been described in detail in two previous papers (Hecht and Wolf, 1928-29; Wolf, 1932-33). With the help of Fig. 1 the nature of the experimental procedures can be made out. The striped pattern underneath the inclined creeping plane upon which the bee crawls can be moved sidewise. For the *visual acuity* test the pattern plates were made of stripe systems of different widths, and for each width of stripe the necessary intensity given by source B was determined for the first noticeable response of the bee. In testing *intensity discrimination*, only one wide stripe width was used, and for each given illumination  $I$  furnished by source A the necessary intensity of  $\Delta I$  for minimal response furnished by source B was determined; and thus one curve was obtained with that one width of stripe. In com-

binning *both* visual acuity and intensity discrimination tests, the width of the stripes had to be altered and a complete curve for intensity discrimination worked out for each width of stripes. Altogether, ten different widths of stripes were used. The larger ones were made by putting opaque black paper strips on the lower surface of opal glass plates, and covering the paper stripes from underneath with a clear glass plate to press them tight against the opal glass. The finer stripes were machine-engraved on glass and have the opaque areas filled in with black printer's ink. These plates were covered with opal plates

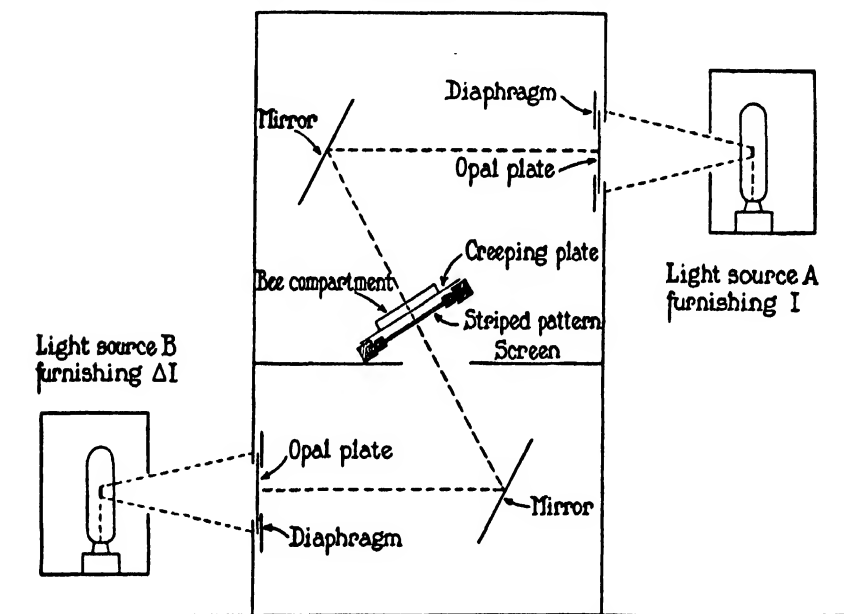


FIG. 1. Diagram of apparatus for measuring the visual intensity discrimination of the bee

in the same manner. During use they were fitted into the movable frame (Fig. 1) so that the opal plate was on top nearest to the bee. The visual acuities determined by these plates depend upon their distance from the eye of the bee. It was arranged in these experiments that the average distance of the upper surface of the opal plate from the bee's head was the same as in the visual acuity experiments carried on some years ago (Hecht and Wolf, 1928-29). The distance to be considered in the intensity discrimination tests is not the distance from the center of the bee's eye to the pattern plate,

but to the upper surface of the *opal* plate, because the opal plate acts as a diffusing screen giving an image of the bars of the same width on its upper surface. The average distance, which varies slightly from plate to plate and from bee to bee, was 17.3 mm., which corresponds to the value we had in the visual acuity experiments (Hecht and Wolf, 1928-29). Consequently the values for the visual angles subtended by the bars, and the corresponding visual acuities, were in the two cases identical. In Table I the dimensions of the bars and spaces, the resulting visual angles, and the reciprocals of the visual angles repre-

TABLE I  
*Designations and Properties of Pattern Plates Used in the Experiments*

Designation	Width of bar	Visual angle subtended by bar	Visual acuity $\times 10^4$
	<i>mm.</i>	<i>min.</i>	
A	20.0	2949.0	3.40
B	12.4	2136.0	4.68
C	9.4	1710.0	5.85
D	6.3	1200.0	8.33
F	3.2	630.0	15.87
G	1.27	252.0	39.68
I	0.635	126.0	79.37
K	0.423	84.0	119.0
L	0.363	72.0	138.9
M	0.318	63.0	158.7

sending the visual acuities, are given. As in the case of ophthalmological practice, a *visual angle* of 1 minute corresponds to a *visual acuity* of 1.

The experiments were carried on exactly in the same manner as the previous ones. Over a desirable range of intensities ( $I$ ), the necessary respective values for  $\Delta I$  were determined, and thus intensity discrimination curves were obtained for each set of stripes. With source *A* turned on, the upper surface of the opal plate will look evenly illuminated ( $I$ ); with light from source *B* in addition, the spaces in between these stripes will have a brightness  $I + \Delta I$ ; so that by appropriately changing the intensity given by source *B* the minimal difference in brightness between the two line systems can be determined which gives a just noticeable response of the bee to a lateral displace-

ment of the line systems. Each point plotted on the curves (Fig. 2) represents an average of ten tests with different individuals. Besides the formerly derived intensity discrimination curve, nine new ones were obtained. Altogether 1,120 bees were tested at different intensities and widths of stripes. The results are given in 112 plotted points, to which the best fitting curves were fitted for the different visual acuity levels.

### III

#### RESULTS

The experiments were carried on during the latter part of the summer and fall of 1932, with the same apparatus used during the earlier part of the year for the first determination of intensity discrimination by the bee. The light sources at their different positions and the intensity values given at different diaphragm openings were re-calibrated. Then the intensity discrimination at different visual acuities was studied in the order of the magnitudes of the bars from the largest width to the smallest, in the same order as indicated in Table I.

The data obtained for the different widths of stripes are given in Tables II to X. The values of  $\Delta I$  and of  $\Delta I/I$  in the tables are *mean* values for the total numbers of individuals tested at each  $I$ , with the probable errors of  $\Delta I/I$  and of  $\Delta I$  computed according to Peter's formula.

The values for  $\Delta I/I$  at each level of visual acuity vary in a significant manner with illumination. For each case, at low intensities  $\Delta I/I$  is greatest; it decreases smoothly as the illumination is increased. At the highest illuminations the values for intensity discrimination at different visual acuities are about identical. At lower illuminations they differ significantly according to the dimensions of the striped pattern used and to the visual angles subtended by the different stripe systems. The probable error of  $\Delta I/I$  decreases with increasing  $I$  for each visual acuity in the same way as does  $\Delta I/I$  itself; and as  $\Delta I$  increases with increasing  $I$ , the probable error of  $\Delta I$  increases.

In Fig. 2 the data are set out graphically. The points plotted are the mean values for all individuals tested at the respective intensities.

# TABLES OF DATA

Mean values for intensity discrimination at different visual acuities and at different intensities measured in millilamberts with their P.E. (number of observations = 10 in each case).

I Millilamberts	$\Delta I$ Millilamberts	$\Delta I/I$
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## TABLE II

Stripes B, 12.4 mm. wide. Visual acuity  $\times 10^4 = 4.68$

0.107	0.528 $\pm$ 0.021	4.924 $\pm$ 0.192
0.134	0.451 $\pm$ 0.010	3.374 $\pm$ 0.076
0.166	0.501 $\pm$ 0.012	3.010 $\pm$ 0.080
0.302	0.594 $\pm$ 0.011	1.968 $\pm$ 0.038
0.417	0.784 $\pm$ 0.015	1.880 $\pm$ 0.035
0.695	1.056 $\pm$ 0.019	1.245 $\pm$ 0.027
1.031	0.951 $\pm$ 0.048	0.922 $\pm$ 0.035
1.439	1.199 $\pm$ 0.044	0.833 $\pm$ 0.028
2.711	1.581 $\pm$ 0.023	0.583 $\pm$ 0.0088
3.900	1.630 $\pm$ 0.020	0.418 $\pm$ 0.0052
6.792	2.821 $\pm$ 0.083	0.415 $\pm$ 0.0129
13.87	3.430 $\pm$ 0.221	0.247 $\pm$ 0.0136
34.68	8.445 $\pm$ 0.298	0.243 $\pm$ 0.0088
110.02	21.490 $\pm$ 0.353	0.195 $\pm$ 0.0032

## TABLE III

Stripes C, 9.4 mm. wide. Visual acuity  $\times 10^4 = 5.85$

0.166	0.760 $\pm$ 0.018	4.567 $\pm$ 0.113
0.214	0.710 $\pm$ 0.017	3.319 $\pm$ 0.075
0.302	0.763 $\pm$ 0.030	2.527 $\pm$ 0.094
0.695	1.175 $\pm$ 0.029	1.690 $\pm$ 0.042
1.439	1.447 $\pm$ 0.019	1.005 $\pm$ 0.014
2.711	1.675 $\pm$ 0.035	0.617 $\pm$ 0.013
5.261	3.068 $\pm$ 0.093	0.583 $\pm$ 0.018
9.572	3.732 $\pm$ 0.098	0.391 $\pm$ 0.0099
19.96	6.305 $\pm$ 0.116	0.316 $\pm$ 0.0057
34.68	8.630 $\pm$ 0.119	0.249 $\pm$ 0.0035
55.04	13.400 $\pm$ 0.234	0.243 $\pm$ 0.0042

## TABLE IV

Stripes D, 6.3 mm. wide. Visual acuity  $\times 10^4 = 8.33$

0.214	0.948 $\pm$ 0.025	4.433 $\pm$ 0.106
0.417	0.992 $\pm$ 0.019	2.381 $\pm$ 0.052
0.695	1.169 $\pm$ 0.015	1.681 $\pm$ 0.034
1.439	1.663 $\pm$ 0.026	1.155 $\pm$ 0.019
2.711	1.878 $\pm$ 0.033	0.693 $\pm$ 0.0099
5.261	3.356 $\pm$ 0.123	0.638 $\pm$ 0.0238
19.960	6.292 $\pm$ 0.206	0.315 $\pm$ 0.0117
81.460	20.670 $\pm$ 0.310	0.254 $\pm$ 0.0039

TABLES OF DATA—*Continued*

I Millilamberts	$\Delta I$ Millilamberts	$\Delta I/I$
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TABLE V

Stripes F, 3.2 mm. wide. Visual acuity  $\times 10^4 = 15.87$ 

0.302	$1.229 \pm 0.017$	$4.068 \pm 0.055$
0.417	$1.247 \pm 0.038$	$2.991 \pm 0.080$
0.695	$1.244 \pm 0.023$	$1.790 \pm 0.033$
1.031	$1.573 \pm 0.043$	$1.506 \pm 0.042$
2.711	$2.609 \pm 0.083$	$0.962 \pm 0.036$
6.792	$3.732 \pm 0.166$	$0.549 \pm 0.024$
19.960	$6.831 \pm 0.151$	$0.342 \pm 0.0075$
34.680	$10.310 \pm 0.178$	$0.297 \pm 0.0054$
81.460	$18.920 \pm 0.296$	$0.232 \pm 0.0037$

TABLE VI

Stripes G, 1.27 mm. wide. Visual acuity  $\times 10^4 = 39.68$ 

0.417	$2.272 \pm 0.020$	$5.451 \pm 0.047$
0.695	$2.214 \pm 0.028$	$3.186 \pm 0.040$
1.031	$2.527 \pm 0.087$	$2.451 \pm 0.045$
2.153	$3.966 \pm 0.090$	$1.331 \pm 0.043$
3.900	$3.989 \pm 0.137$	$1.023 \pm 0.037$
9.572	$6.266 \pm 0.118$	$0.654 \pm 0.012$
13.870	$5.958 \pm 0.214$	$0.430 \pm 0.015$
34.680	$10.650 \pm 0.257$	$0.307 \pm 0.0083$
70.270	$17.380 \pm 0.293$	$0.247 \pm 0.0041$

TABLE VII

Stripes I, 0.635 mm. wide. Visual acuity  $\times 10^4 = 79.37$ 

1.031	$5.719 \pm 0.200$	$5.547 \pm 0.138$
1.439	$5.691 \pm 0.186$	$3.955 \pm 0.129$
2.711	$6.426 \pm 0.120$	$2.370 \pm 0.060$
3.900	$6.869 \pm 0.254$	$1.761 \pm 0.079$
6.792	$10.860 \pm 0.375$	$1.465 \pm 0.058$
9.572	$10.910 \pm 0.200$	$1.140 \pm 0.020$
19.960	$13.630 \pm 0.203$	$0.683 \pm 0.010$
34.680	$15.150 \pm 0.189$	$0.437 \pm 0.0052$
61.720	$18.630 \pm 0.361$	$0.302 \pm 0.0059$
110.020	$29.570 \pm 0.299$	$0.270 \pm 0.0025$

TABLES OF DATA—*Concluded*

I Millilamberts	$\Delta I$ Millilamberts	$\Delta I/I$
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TABLE VIII

Stripes K, 0.423 mm. wide. Visual acuity  $\times 10^4 = 119.0$ 

2.711	11.600 $\pm 0.296$	4.280 $\pm 0.111$
3.900	13.700 $\pm 0.197$	3.514 $\pm 0.047$
5.261	15.590 $\pm 0.313$	2.394 $\pm 0.060$
9.572	16.030 $\pm 0.279$	1.675 $\pm 0.030$
13.870	16.630 $\pm 0.271$	1.199 $\pm 0.019$
19.960	19.190 $\pm 0.273$	0.962 $\pm 0.014$
34.640	23.100 $\pm 0.313$	0.666 $\pm 0.0091$
55.040	27.110 $\pm 0.477$	0.493 $\pm 0.0087$
110.020	30.340 $\pm 0.350$	0.276 $\pm 0.0032$

TABLE IX

Stripes L, 0.363 mm. wide. Visual acuity  $\times 10^4 = 138.9$ 

3.900	21.190 $\pm 0.558$	5.432 $\pm 0.139$
5.261	21.550 $\pm 0.454$	4.095 $\pm 0.085$
9.572	22.950 $\pm 0.443$	2.397 $\pm 0.043$
13.870	22.900 $\pm 0.378$	1.651 $\pm 0.025$
19.960	23.520 $\pm 0.465$	1.178 $\pm 0.026$
27.420	30.640 $\pm 0.355$	1.117 $\pm 0.013$
34.680	32.510 $\pm 0.355$	0.937 $\pm 0.010$
55.040	35.610 $\pm 0.744$	0.647 $\pm 0.011$
110.020	55.500 $\pm 0.795$	0.504 $\pm 0.010$

TABLE X

Stripes M, 0.318 mm. wide. Visual acuity  $\times 10^4 = 158.7$ 

13.87	58.020 $\pm 0.998$	4.183 $\pm 0.071$
19.96	60.400 $\pm 1.156$	3.026 $\pm 0.058$
27.42	61.730 $\pm 0.900$	2.251 $\pm 0.039$
34.68	67.210 $\pm 1.238$	1.938 $\pm 0.030$
81.46	94.730 $\pm 0.383$	1.163 $\pm 0.0047$
110.02	93.980 $\pm 0.840$	0.854 $\pm 0.0076$

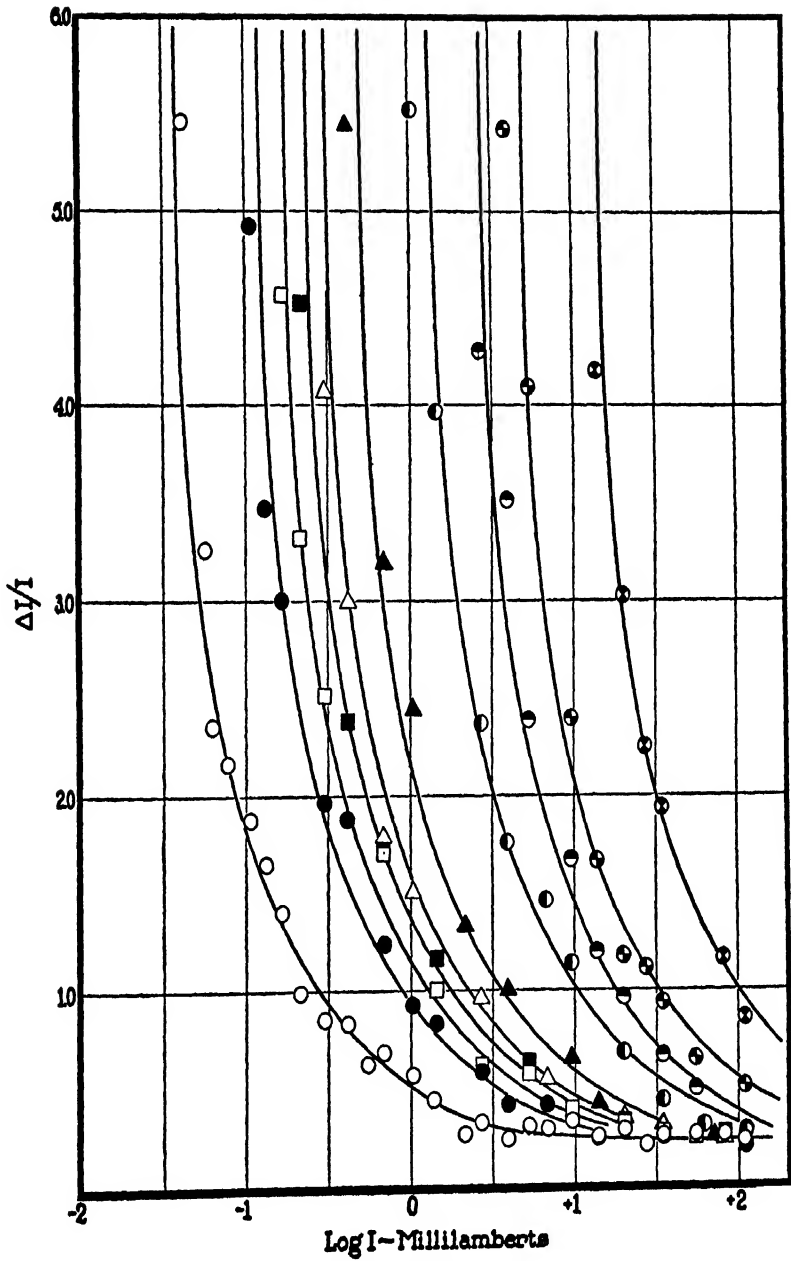


FIG. 2. Relation between intensity discrimination and illumination at different visual acuities. Each curve represents an intensity discrimination curve for a different visual acuity; the points represent averages of ten bees tested at each illumination.



The first curve (open circlets) is the intensity discrimination graph published previously, where the width of the bars of the pattern was chosen great enough so that the bees were able to react to the bars at any illumination used. The nine other curves are intensity discrimination curves for increasingly greater visual acuities. These curves show that with greater visual acuities, intensity discrimination gets worse at lower illuminations, whereas at higher intensities the discrimination power is improved in practically the same course as in our first experiments—until at the highest illuminations visual acuity does not interfere with the discrimination, save for the two smallest sizes of striped patterns, for which the curves do not come down to the same level within the range of intensities used. This, however, is due only to the fact that one is practically limited in obtaining high enough intensities for  $I$  and  $\Delta I$ , providing in the extreme case values for  $\Delta I/I$  which would correspond to the minimal value found for smaller visual acuities.

#### IV

The ten curves for intensity discrimination at different visual acuities are in their general course identical, with a progressive shift to greater values on the  $\log I$  scale in Fig. 2. This suggests a significant interrelation between visual acuity and intensity discrimination. The gradual shift of the curves to the right is not so evenly spaced in relation to the width of stripe as one might perhaps expect. The shift corresponds much more to the difference in abscissa values found for the positions of the points on a visual acuity curve for the bee. And so the question arises, whether one is able to construct a series of visual acuity curves from the intersections of the intensity discrimination curves with a given ordinate value of  $\Delta I/I$ .

In Fig. 3, four such reconstructions of visual acuity curves are given. The first curve (white circles) is the visual acuity curve obtained by Hecht and Wolf (1928–29). The second curve (black circles) is a visual acuity curve constructed from the intersections of the intensity curves with the ordinate  $\Delta I/I = 3.0$ ; the third (open squares) is at  $\Delta I/I = 2.0$ ; the fourth (black squares) at  $\Delta I/I = 1.0$ ; and the fifth (half circles) at  $\Delta I/I = 0.5$ . The curves fitted to the points are the

same as the original curve, only shifted to the right. The fit is reasonably good, and is sufficient to show that the curves are essentially identical. The graph shows that with  $\Delta I/I$  getting smaller the fit of the curve becomes better, which is only to be expected because at higher intensities more precise settings can be made, as the bees give sharper and more definite responses.

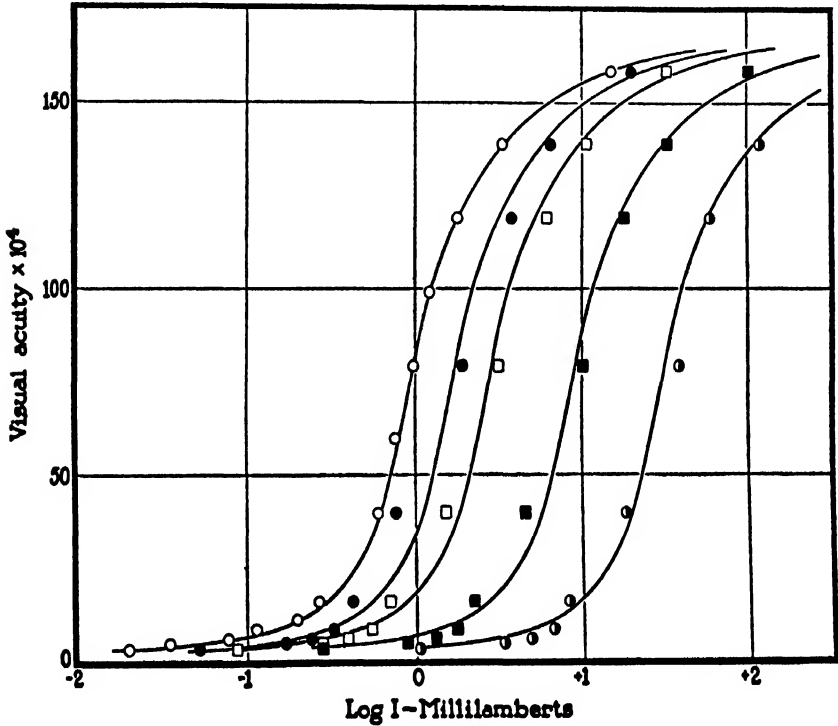


FIG. 3. Visual acuity curves reconstructed from the data given in Fig. 2. The first curve is the original visual acuity curve for the bee; the second is reconstructed for  $\Delta I/I = 3.0$ ; the third for  $\Delta I/I = 2.0$ ; the fourth for  $\Delta I/I = 1.0$ ; and the fifth for  $\Delta I/I = 0.5$ .

A visual acuity curve provides the magnitudes of  $I$  corresponding to successively decreased width of stripe at a given magnitude of  $\Delta I/I$ ;  $\Delta I$  measures nearly enough, for each value of  $I$ , the increase of intensity which is necessary to bring into action a certain constant number of additional receptors. Consequently we should expect that, for decreasing magnitudes of  $\Delta I/I$  (that is, increasing values of  $I$  and  $\Delta I$ ), the visual acuity curve must shift toward the right, that is,

toward higher levels of  $I$ : but that its *form* should not be changed. This is evident in Fig. 3.

For visual acuity tests in which the bee reacts to a pattern of alternately black and illuminated stripes, we may say that  $\Delta I/I$  equals infinity, which means  $I = 0$ . If we intend to obtain from our different intensity discrimination curves a curve which would be identical with the first of the five curves in Fig. 3, we have to take the values on the curves at a very large magnitude of  $\Delta I/I$ . It can be shown that the points of the original visual acuity curve (Hecht and Wolf, 1928-29) and the curve at  $\Delta I/I = 6.0$  fall very close together, and presumably on extrapolating to still greater values for  $\Delta I/I$  the two curves would actually coincide. No attempt has been made to show this graph because the intensity discriminations at even values of  $\Delta I/I = 4.0$  is pretty uncertain, so that one cannot put too much emphasis on the observations made beyond that point. Some points of the desired visual acuity curve ( $\Delta I/I > 6.0$ ) would fall onto the left of the original visual acuity curve where  $\Delta I/I$  was infinite, and that would be practically impossible; consequently all such values only indicate that at such low illuminations settings of the apparatus accurate enough to be reliable cannot be made.

The reproducibility of the visual acuity curves from the intensity discrimination data, in view of the delicacy of measurement and of curve fitting, is of some special interest. The data collected in the  $\Delta I/I$  curves were obtained from different animals of the same colony. Each point on the curves represents a mean value for ten different individuals. It has been emphasized previously that on account of the genetic uniformity of the members of a colony of bees, there is no special need for establishing a complete intensity discrimination curve with one single individual. It has been shown that frequent handling of the bees for repeated observations upon one worker actually interferes a good deal with the accuracy of the determinations. It is furthermore of interest to see the reproducibility of the visual acuity curve by comparison with that obtained using bees of a different colony some years ago (Hecht and Wolf, 1928-29). This indicates not only that a fairly high degree of uniformity as to reactions exists within the members of one single colony, but also among different colonies of commercial lines of bees.

The visual acuity computed from the width of the stripes and the distance of the pattern from the bee's eye is in surprisingly precise agreement with the observations. It was mentioned before that for these determinations the distance of 17.3 mm. from the center of the bee's eye to the upper surface of the opal cover-plate had to be considered, and not the distance to the actual stripes which is 18.7 mm. If one attempts to fit the original visual acuity curve to the greater visual acuity values based upon the distance to the bars, the fit of the curves at once becomes inadequate. This indicates that for one set of determinations of visual acuity values at different illuminations, only one best fitting curve can be drawn.

#### SUMMARY

1. Bees respond by a characteristic reflex to a movement of their visual field. By confining the field to a series of parallel stripes of two alternating different brightnesses it is possible to determine for any width of stripe, at any brightness of one of the two sets of stripes, the brightness of the second at which the bee will first respond to a displacement of the field. Thus the relations between visual acuity and intensity discrimination can be studied.

2. For each width of stripe and visual angle subtended by the stripe the discrimination power of the bee's eye for different brightnesses was studied. For each visual acuity the intensity discrimination varies with illumination in a characteristic, consistent manner. The discrimination is poor at low illuminations; as the intensity of illumination increases the discrimination increases, and reaches a constant level at high illuminations.

3. From the intensity discrimination curves obtained at different visual acuities, visual acuity curves can be reconstructed for different values of  $\Delta I/I$ . The curves thus obtained are identical in form with the curve found previously by direct test for the relation between visual acuity and illumination.

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# THE VARIABILITY OF INTENSITY DISCRIMINATION BY THE HONEY BEE IN RELATION TO VISUAL ACUITY

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It is necessary to produce an intensity of illumination ( $I + \Delta I$ ) in one component of a series of alternating bars differently illuminated, the intensity of illumination of the other set being  $I$ , in order to call forth a response in a creeping bee when the visual field so composed is moved to one side. With wide stripes the variability of ( $I + \Delta I$ ) depends upon the intensity of illumination  $I$  in exactly the same manner as does ( $I + \Delta I$ ). When  $I$  is high, and thus  $\Delta I$ , the standard deviation of  $\Delta I$  is high; it decreases as a simple power function of decreasing  $I$ , until a very low intensity is reached (*cf.* Wolf, 1932-33*a*). These determinations were made with one particular width of bars (20 mm.) constituting the illuminated pattern in the substratum upon which the bee creeps (Wolf, 1932-33*b*). It is important to consider the manner in which the variability of  $\Delta I$  may be affected according to the widths of the bars of light. Variability is defined as the quantitative dependence of the scatter of the determinations upon the magnitudes of the independent variable—in this case, the illumination  $I$  (*cf.* Crozier, 1929; Crozier and Pincus, 1931-32; etc.).

Clearly, the variability of the measurements, considered in this way, has an intimate bearing upon the automatic demonstration of internal self-consistency in the data, thus providing a control upon the consistency of the observer's performance; and, since it gives a means of estimating the variability of responsiveness of the tested organism, it is also important for questions as to the rôle of "pattern," "form discrimination," and several related topics. In this case, the pattern is simple and its properties may be suggestive. The excitation of the bee by movement of or in its visual field is clearly and definitely apparent by a reaction—a change of direction of the bee's path—when the animal is creeping geotropically upon an inclined surface. The

immediate cause of the response is given not by the movement of the bee, but by a movement of the diversely illuminated pattern above which it creeps. The bee does not react to motion of the stripes if it is not moving.

Excitation must depend upon the passage of a certain number of ommatidia from the less excited to the more excited state (or the reverse, or both) while the bee is creeping. This must depend largely upon the sizes of the alternate bars illuminated in a contrasting manner. Since the total area of the field respectively covered by the brighter and by the less bright bars which alternate in position is independent of the width of the bars, the excitation must be a function of the frequency of occurrence of divisions between the two areas, since the greater frequency of such divisions per unit area the greater the chances of a given ommatidium passing from less excited to more excited state. We found previously (Wolf, 1932-33*a*) that the variation of  $\Delta I$  increased as a power function of  $I$ , with given constant width of stripe (20 mm.). With bars of narrower width one must then expect that at given intensity of illumination the excitation must be higher—the visual angle being smaller—and consequently the variation of  $\Delta I$  must be expected to be higher, reducing with continuing increase of illumination to a certain magnitude dependent upon maximal excitation of all the ommatidia. With still narrower bars, this process must be expected to continue. This would of itself be enough to show that the observed variation of  $\Delta I$  is connected with a property of the bee, rather than a matter of “experimental error.”

It was found with bars of 20 mm. width that the P.E. of  $(\Delta I/I)$  declined hyperbolically with  $\log I$ . In terms of the reasoning thus far outlined, we expect this relationship to be apparent with stripe systems of narrower widths, but that with successively narrower stripes the curve of hyperbolic descent of P.E.  $(\Delta I/I)$  with increasing  $\log I$  should move successively toward higher values of  $\log I$ . For the present series of measurements (Wolf, 1932-33*b*) Fig. 1 demonstrates that this relationship is indeed obeyed. The data are contained in the preceding paper by Wolf (1932-33*b*). As there explained, the readings are averages of ten determinations of  $\Delta I$ , and the probable errors of these averages. Since the number of observations is constant in each set, the use of a probable error of the mean is merely a matter

of convenience, and in no way affects quantitatively or qualitatively the character of the subsequent reasoning; we are quite well aware that other methods of expressing the scatter of observations might be preferable for other purposes. P.E. ( $\Delta I/I$ ) decreases with increasing  $I$  in precisely the same manner as does  $\Delta I/I$  itself (Wolf, 1932-33*b*). It was concluded previously (Wolf, 1932-33*a*) that the quantitative

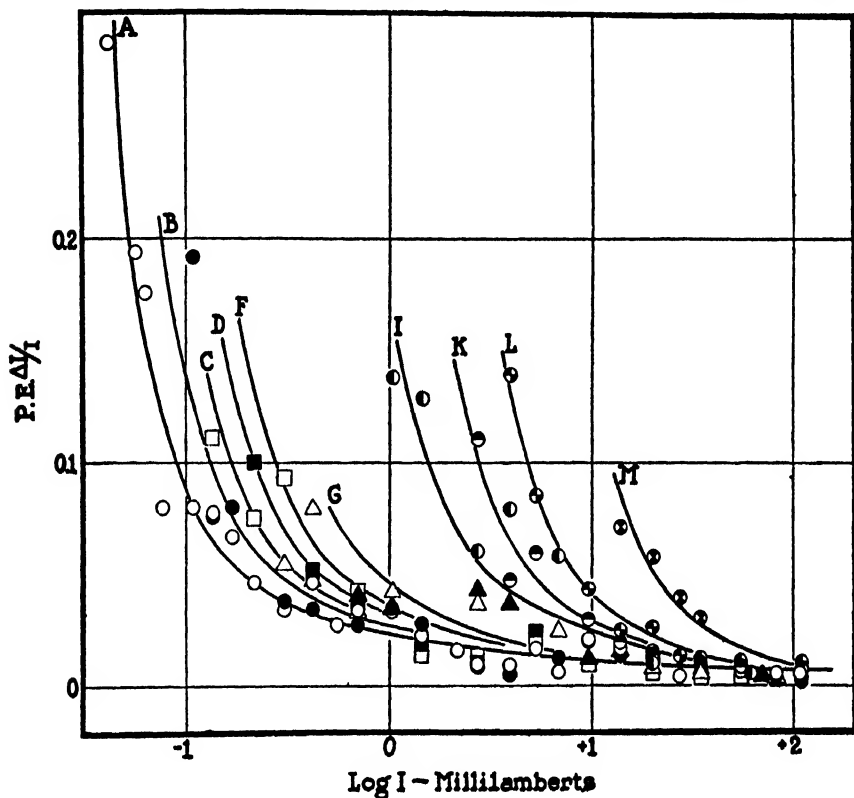


FIG. 1. Probable errors of the ratios ( $\Delta I/I$ ) as a function of  $\log I$ , for stripes of different widths, A to M, progressively narrower. The widths of stripes, and the corresponding visual angles, are given in an accompanying paper (Wolf, 1932-33*b*).

level of intensity discrimination—with stripes of given width—determines or limits the variation in the measure of this discrimination. From Fig. 1 it is apparent that with stripes of narrower and narrower widths the P.E. of ( $\Delta I/I$ ) is higher and higher at given intensity of illumination; put in another way, the narrower the stripe, the higher the intensity of illumination required to produce a given variation in



( $\Delta I/I$ ). This is completely consistent with the thought that the general intensity of excitation directly determines the magnitude of the variation in reactiveness for the threshold response which is the basis of the measurements.

In one sense, this is more simply seen if one considers that, at given magnitude of  $I$ ,  $\Delta I$  is inversely proportional to the visual angle subtended by the width of one of the stripes—that is, by the distance between the lines of division of the illuminated areas (visual angle is of course the reciprocal of the visual acuity); the curve relating  $\Delta I$  with visual angle is almost exactly hyperbolic, so that  $\Delta I$  is almost directly proportional to the visual acuity. At given magnitude of intensity, P.E.  $\Delta I$  increases with  $\Delta I$ , that is, with decreasing visual angle. This is consistent with the idea that the observed variation of  $\Delta I$  tends to be progressively restricted as the fineness of discrimination (decreasing value of  $\Delta I$ ) becomes greater.

For the stripes of greatest width used (20 mm., *A*, Fig. 1)  $\Delta$  (P.E.  $\Delta I$ ) / (P.E.  $\Delta I$ ) is directly proportional to  $\Delta I/I$ , until, at a very low level of intensity, P.E.  $\Delta I$  increases somewhat, owing presumably in part to lack of nicety in the adjustment of the apparatus (that is, of  $I + \Delta I$ ) by the observer. With narrower illuminated bars (Fig. 2) this secondary source of increase in P.E.  $\Delta I$  naturally appears at higher and higher intensities, until, beyond the inflection point in the curve for visual acuity at any intensity of illumination (*cf.* Wolf, 1932-33*b*; Fig. 2), the curvature of the departures of P.E.  $\Delta I$  from the limiting line connecting  $\log$  P.E.  $\Delta I$  with  $\log I$  assumes a somewhat different form. With stripes of still narrower widths, the visual acuity at given intensity of illumination becomes higher and declines less rapidly for a given increase of illumination; consequently, the curves for departure of  $\log$  P.E.  $\Delta I$  from the limiting line descend less rapidly to this line.

A fundamental problem arising in relation to the sensory effects, or the effects upon behavior, of the seen movement of a patterned field, has to do with the question as to whether effects induced are of purely peripheral, that is, sensory, origin; or whether the character of the pattern as a pattern is intrinsically significant. This question can be determined most directly, not by the consideration of picturesque differences in forms of pattern, but by the close analysis of the influence of simple changes in pattern of which the possible sensory consequences

may be determined and checked. In the present instance we have to do with a visual field in which the differences experimentally produced concern, not modifications of the relative amounts of brighter and less bright surface, but changes in the frequency per unit area of divisions

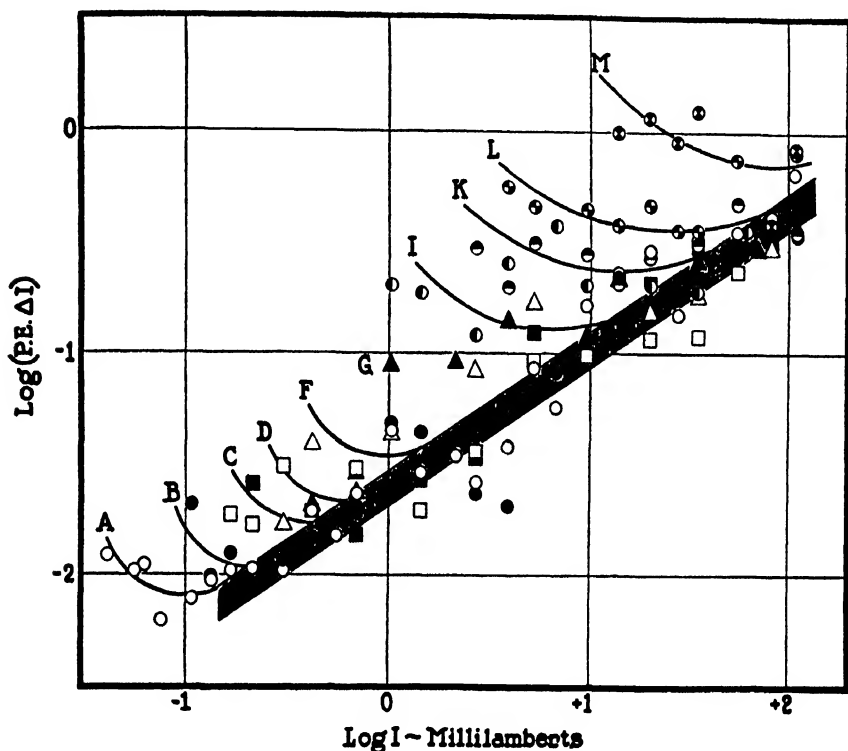


FIG. 2. Probable error  $\Delta I$  considered as a power function of  $I$ . For stripes of greatest width (A) the wide bar gives the limiting line (cf. Wolf, 1930; 1932-33a); with stripe systems of progressively narrower widths, to M, departures from this limiting line are increasingly more extensive. This is discussed in the text. For the stripes of a width corresponding to the location of the inflection point in the curve for visual acuity (Wolf, 1932-33b) the variation is much more extensive than in the data obtained with the other stripes; for these observations, G, no curve is drawn.

between brighter and less bright; this means, that with given frequency (that is, with the total number) of demarcation lines encountered by the ommatidial surface, the excitation of an ommatidium, so far as concerns the elicitation of the threshold response which is the basis of measurement, must depend upon the passage of ommatidia from

a condition of illumination by the brighter or the less bright bars to the reverse. The visual acuity curves determined by this method, and the intensity discrimination curves upon which they are based, can be understood on the assumption (*cf.* Hecht and Wolf, 1928-29) that excitation is determined at given intensity of illumination by the number of ommatidia stimulated, and fundamentally by the distribution of thresholds for photic excitation as a function of intensity among the total population of ommatidia. We find here that not only do the data upon intensity discrimination retain a consistent character regardless of the width of stripe (that is, detail of the pattern) but, what is more surprising, that the variation in the measurement of  $\Delta I$ , considered as a function of  $I$ , can also be understood completely as to its form, on the assumption that the relative change of the standard deviation of  $\Delta I$ , when  $I$  is altered, is fundamentally directly proportional to the relative change of intensity, but with a modification of the fundamental limiting graph from which this statement is derived which becomes apparent when, with narrower and narrower illuminated bars, the increasing frequency of excitation, due to the increased frequency of transitions from brighter to less bright illuminations upon the bee's eye, determines at a given intensity of illumination a higher intensity of excitation. It is conceivable that, from the character of these departures from the limiting line in the graph of P.E.  $\Delta I$  against  $I$ , estimation could be made of the manner in which narrowing the widths of bars to the point beyond the inflection point in the visual acuity graph serves to bring about increased excitation; in other words, narrowing the bars increases the frequency of excitation (over a relatively gross interval of time); increasing  $I$  increases the total intensity of a single excitation. It is to be remembered, again, that in these measurements one deals with a threshold response, which does not depend upon the speed of the translational displacement of the seen pattern, at least within rather wide limits not exceeded in securing the actual readings.

#### SUMMARY

Variation in the determined magnitudes of the difference in brightness between alternating members of a system of stripes requisite for the elicitation of a threshold response in bees shows that the intensity

of excitation, as a function of width of stripe and of intensity of illumination, is determined by the intensity of illumination and by the frequency of occurrence of divisions between bright and less bright bars. The variation of  $\Delta I$  is limited by the intensity of excitation, so that the curves relating P.E. ( $\Delta I/I$ ) have the same form in relation to  $I$  as do the curves for  $\Delta I/I$ . The limiting rule according to which P.E.  $\Delta I$  is a power function of  $I$  for stripes of maximum usable width is departed from more and more markedly, for lower intensities, as narrower stripes are employed.

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# THE DIFFUSION COEFFICIENT OF CRYSTALLINE TRYPSIN

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A simplified method for the determination of diffusion coefficients, and the use of the latter in the calculation of molecular radii and weights, have been described by Northrop and Anson (1). The use of the method has been extended to the study of crystalline pepsin and crystalline trypsin, in an attempt to separate by fractional diffusion (2) molecules responsible for the proteolytic activity from those containing protein nitrogen. The fact that no fractionation could be ascertained by this method, since the diffusion of proteolytic activity and of protein nitrogen ran parallel, was interpreted as evidence that the former is an integral property of the protein molecule. The determination of the diffusion coefficient of crystalline trypsin is here reported in detail.

*Assembly of the Apparatus.*<sup>1</sup>—The apparatus was assembled somewhat differently from the description given by Northrop and Anson (1) and is illustrated in Fig. 1. The outer vessel measured  $6 \times 20$  cm. The siphon tube was 2 to 3 mm. inside diameter, so that the fixed error due to the fact that free diffusion does not take place into the liquid in the tube was negligible. The outer vessel was held by a large condenser clamp which in turn was mounted on a ring-stand with tripod base, two of whose feet were fitted with thumb screws to facilitate leveling of the apparatus. The outer vessel was partially submerged, as shown, in a large water-bath set up in a room in which the temperature was maintained at  $5^\circ \pm 1^\circ\text{C}$ . During the day when the room was used extensively and the temperature tended to rise, it was necessary to control the temperature of the bath by the addition of ice. Care must be taken that the apparatus is mounted where it will not be subjected to vibrations or sudden jars. To this end, the bath was not stirred mechanically. Also, to avoid disturbances during the withdrawal of samples, a rubber tube of small diameter and about 2 feet long was permanently attached to the siphon tube during an experiment, and the samples were withdrawn by

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<sup>1</sup> This modified assembly was devised by Dr. Moses Kunitz.

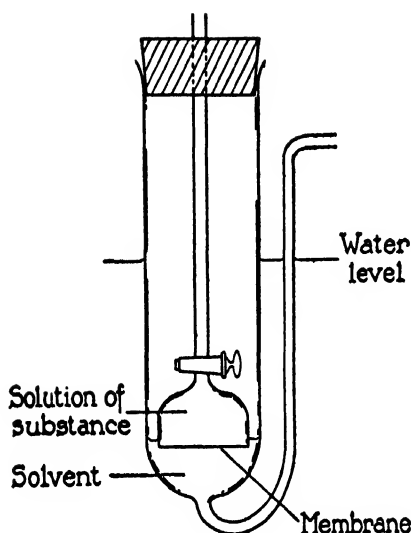


FIG. 1. Apparatus for the determination of diffusion coefficients.

surface of the liquid. There is a fixed error here due to the fact that diffusion does not take place into that portion of the liquid which is above the level of the membrane. However, a simple calculation showed that this error could be safely disregarded. The plane of the membrane was made approximately parallel to that of the surface of the solvent by manipulation of the condenser clamp and the final leveling adjustment obtained by means of the thumb screws in the tripod base of the ring-stand.

Equilibration was allowed to proceed for 2 hours to overcome any possible differences in salt concentration between the two solutions, the outer solution being then withdrawn and discarded since it contained only a small amount of trypsin. The apparatus was rinsed by introducing 20 cc. of solvent, which was likewise drawn off and discarded. A fresh sample of solvent was then introduced and diffusion was allowed to proceed for the desired length of time. The process of rinsing was repeated each time a sample was withdrawn for analysis, all rinsing solutions being discarded.

At the conclusion of an experiment, the cell was washed out thoroughly with distilled water and kept in contact with the same solvent during the interval between determinations, since changes arise in the cell constant if drying occurs.

*Calibration of the Apparatus.*—Two cells were used in these experiments, both of which had membranes made from Jena glass filter

suction into small flasks. Fresh solvent was introduced through a notch in the side of the stopper.

In setting up an experiment with solutions of crystalline trypsin, the diffusion cell was filled through the stop-cock by means of a capillary pipette, since the high viscosity of the solution made filling by suction through the porous membrane very slow. A small amount of mercury was placed in the tube above the stop-cock to safeguard against leakage. The desired amount of solvent was placed in the outside vessel (20 cc. was used throughout these experiments), and the cell mounted in position and lowered until the membrane was 1–2 mm. below the

discs.<sup>2</sup> The internal volume was determined by weighing dry and filling with water, very close checks being obtained by this method. The values found were 24.55 cc. for Cell 2 and 5.09 cc. for Cell 4. A cell was used with a small internal volume (Cell 4) relative to the volume of solvent outside, in order that the fraction of the original solution which diffused across the membrane should be as large as possible.

The cell constant,  $K$ , was determined with carbon monoxide hemoglobin as the known substance, using the value for the diffusion coefficient,  $D$ , reported by Northrop and Anson; namely, 0.0420 cm.<sup>2</sup> per day. Analysis was by measurement of total nitrogen by the micro Kjeldahl method. The results were checked by colorimetric determinations. The values of  $K$  were calculated from the extended form of the diffusion equation previously developed (1).

$$(1) \quad D = \frac{2.3 K v_1 v_2}{(v_1 + v_2) t} \log \frac{v_2 S}{v_2 S - (v_1 + v_2) Q}$$

This equation was used throughout these experiments.  $K$  for Cell 2 was found to be 0.054 and for Cell 4, 0.0315.

*Preparation of Trypsin.*—Two samples of crystalline trypsin were used. One was prepared from several samples of moist trypsin cake of different ages in current use. 8 gm. of cake was dissolved in 30 cc. of 0.1 M acetate buffer at pH 4.0 and the solution was poured into 150 cc. of the same buffer at the boiling point. The mixture was cooled rapidly in running tap water, brought to 0.4 saturation with ammonium sulfate, and filtered through a Schleicher and Schull No. 1450½ filter paper. Ammonium sulfate was added to the filtrate to 0.7 saturation and the resultant precipitate was collected on hard paper on a Buchner funnel. 6 gm. of moist cake was obtained. This was dissolved in 40 cc. of 0.5 saturated magnesium sulfate, 0.1 M acetate buffer, pH 4.0. The solution was used undiluted for Cell 2, and diluted with an equal volume of solvent for Cell 4. The specific activity,  $[T.U.]_{\text{mg.N}}^{4gV}$ , was 133.

<sup>2</sup> The Jenaer Glaswerke, Schott and Genoden, Jena, Germany,—American agent: Fish-Schurman Corporation, 230 East 45th Street, New York,—is now prepared to furnish diffusion membranes made according to the directions of Northrop and Anson and fused onto diffusion cells.



TABLE I

*Diffusion Coefficient of Crystalline Trypsin, 5°C.*

## Experiment 1

Solvent, 0.5 saturated magnesium sulfate, 0.1 M acetate, pH 4.0. Sp. gr. solvent, 5°C., 1.115 gm. cm.<sup>-3</sup>. Viscosity solvent, 5°C., 0.0303 erg sec. cm.<sup>-2</sup>.

Cell No.	Original solution	Time	Q, quantity diffused, of original solution		D	
			By activity	By nitrogen	By activity	By nitrogen
		days	cc.	cc.	in cm. <sup>2</sup> per day	in cm. <sup>2</sup> per day
2						
Internal volume = 24.55 cc.	460 [T.U.] <sup>4gV</sup> / <sub>cc.</sub>	0.156	0.062	—	0.0214	—
		0.708	0.281	0.274	0.0217	0.0212
Cell constant = 0.054	3.45 mg. N/cc.	2.00	0.565*	0.806	0.220	0.0228
4						
Internal volume = 5.90 cc.	Same as for Cell 2, diluted with equal volume of solvent	0.156	0.113	—	0.0231	—
		0.708	0.433	0.382	0.0207	0.0182
Cell constant = 0.0315		2.00	0.756	1.03	0.0201	0.0204
Average.....					0.0218 ±0.0008	0.0207 ±0.0013

## Experiment 2

Solvent, 0.5 saturated magnesium sulfate, sulfuric acid, pH 3.0. Sp. gr. solvent, 5°C., 1.145 gm. cm.<sup>-3</sup>. Viscosity solvent, 5°C., 0.0362 erg sec. cm.<sup>-2</sup>.

2	289 [T.U.] <sup>4gV</sup> / <sub>cc.</sub>	0.50	0.208	0.185	0.0226	0.0202
	4.97 mg. N/cc.	1.00	0.318	0.323	0.0176	0.0178
4	Same as for Cell 2, diluted with equal volume of solvent	0.50	0.332	0.278	0.0217	0.0181
		1.00	0.512	0.532	0.0182	0.0187
Average.....					0.0200 ±0.002	0.0187 ±0.0007

\* In calculating the values of *D* from these figures, a correction has been applied to compensate for the fact that the specific activity of the sample fell about 25 per cent during the latter part of the experiment.

The second sample of trypsin was part of a batch freshly prepared from pancreatic juice and had been subjected to two heat treatments. Its specific activity was 60 [T.U.] $\frac{4gV}{mg.N}$ . It was dissolved to about the same concentration as the first sample, using as solvent, however, 0.5 saturated magnesium sulfate brought to pH 3.0 (just red to methyl orange) by the addition of sulfuric acid.

*Analysis of Samples.*—Protein nitrogen was precipitated from the sample by addition of an equal volume of 20 per cent trichloroacetic acid (in two cases 5 per cent acid was used) and heating just to the boiling point. The coagulum was collected on a hard filter paper and washed with 10 per cent trichloroacetic acid until the filtrate gave a negative test with Nessler's reagent. The precipitate was then washed into a small Kjeldahl flask and the determination carried out as described by Northrop (3).

The determination of proteolytic activity was made by the gelatin viscosity method (4).<sup>3</sup>

The results of the diffusion measurements are summarized in Table I.

#### DISCUSSION

The value of the diffusion coefficient found in Experiment 2 is apparently somewhat less than that in Experiment 1. This discrepancy is attributable to the difference in the viscosity of the solvents used, and disappears when one calculates the molecular radius (including water of hydration) from the equation (1).

$$(2) \quad r = \frac{RT}{6 \pi \eta N D}$$

The figures are  $2.72 \times 10^{-7}$  cm. for Experiment 1 and  $2.5 \times 10^{-7}$  cm. for Experiment 2. This corresponds to an average molar volume for the hydrated protein of 44,700 cm.<sup>3</sup>, calculated from the equation:

$$(3) \quad V = \frac{4}{3} \pi r^3 N$$

The relation of this figure to the one determined by osmotic pressure measurements, and to the extent of hydration calculated from viscosity determinations has already been discussed (2*b*).

<sup>3</sup> These measurements were carried out by Mr. Nicholas Wuest and Miss Margaret R. McDonald.

Examination of Table I shows that the diffusion coefficient of crystalline trypsin was constant, within the experimental error, where (1) two widely different samples of the enzyme were used; (2) two diffusion cells having markedly different characteristics were used; (3) the pH was varied (4.0 in one case, 3.0 in the other); (4) the quantities of original solution diffusing were, respectively, 4.6 per cent, 26.0 per cent, 2.1 per cent, and 14.0 per cent; (5) the result was determined by the rate of diffusion of both proteolytic activity and protein nitrogen. In other words, despite these widely varying conditions, no evidence was found that it is possible, by fractional diffusion, to separate crystalline trypsin into a component responsible for the proteolytic activity and one containing the protein nitrogen. This is direct experimental evidence that the enzyme exists as an integral part of, or in chemical combination with, the protein molecule, and not as part of an adsorption complex. It is also of interest that the molecules formed by spontaneous inactivation of the enzyme have the same dimensions as those of active material, since the specific activity of the partially inactivated sample was not changed by diffusion.

#### SUMMARY

The diffusion coefficient of crystalline trypsin in 0.5 saturated magnesium sulfate at 5°C. is  $0.020 \pm 0.001$  cm.<sup>2</sup> per day, corresponding to a molecular radius of  $2.6 \times 10^{-7}$  cm.

The rate of diffusion of the proteolytic activity is the same as that of the protein nitrogen, indicating that these two properties are held together in chemical combination and not in the form of an adsorption complex.

The author wishes to take this opportunity to thank Dr. John H. Northrop for suggesting these experiments, and Dr. Moses Kunitz for his kind cooperation in conducting them.

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# ANALYSIS OF THE GEOTROPIC ORIENTATION OF YOUNG RATS. VII

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(Accepted for publication, March 17, 1933)

## I

Measurement of the geotropic orientation of young rats creeping upon an inclined surface has lead to a theory of the orientation. For a number of well-inbred lines of rats tested, the relationship between angle of orientation  $\theta$  on the inclined surface and the slope of the surface ( $\alpha$ ) is a specific, characteristic, reproducible property. The curve connecting  $\theta$  with  $\alpha$ , for a standard age of individual (13-14 days), defines the tropistic orientation as a type of performance, in terms of an essential condition of the performance.<sup>1</sup> The curve for each race of rats is compound, comprising three segments; each of these segments behaves as an independent heritable unit in crosses between different races (Crozier and Pincus, 1929-30 *a*, *b*, 1931-32 *e*). The complete curve, extending from a threshold slope of surface to an upper slope beyond which no further significant increase in extent of upward orientation is apparent, is interpreted as resulting from the fact that two elements are concerned in the sensory excitation which results in the oriented creeping. One of these is the magnitude of the gravitational pull experienced by the legs which support and propel the body mass; the second is the frequency with which this gravitational pull acts upon the legs. Each of these elements is directly controlled by the slope of the surface upon which creeping takes place; the speed of

<sup>1</sup> In a discussion of this matter which is not at all clear, von Buddenbrock (1931, pp. 716 *et seq.*) claims that this performance is not tropistic in character, but is a manifestation of a "higher activity;" but he completely ignores the development of the quantitative treatment which derives from the elementary assumption as to the nature of a tropistic performance (Crozier, 1929), namely, that orientation is maintained when excitation is sensorially equivalent upon the two sides of a bilaterally symmetrical organism.

creeping is almost a rectilinear function of  $\log \sin \alpha$ ; this permits differentiation of the curve connecting  $\theta$  with  $\log \sin \alpha$ , in such a manner as to reveal the distribution of thresholds for the excitation of diverse sensory elements (excitation units) as depending upon the slope of surface (Crozier, 1929; Crozier and Pincus, 1929-30*b*, 1931-32*b*, 1931-32*d*). The rôle of the magnitude of the gravitationally induced pull supported by the legs in creeping is tested directly by the imposition of additional loads, and the predictable distortions of the curves connecting  $\theta$  with  $\log \sin \alpha$  are obtained (Crozier and Pincus, 1929-30*b*, 1931-32*d*).

It is a matter of considerable importance to provide an independent test of the propriety of the assumption that the speed of creeping is a separate element in the determination of the magnitude of the excitation. The speed of creeping is taken as the only practicable measure of the frequency with which the legs are called upon to support the gravitationally induced pull. The appropriate test is provided by treatments of the young rats which, without the imposition of additional loads which must be carried, or other rearrangements of leg-supported tensions, will automatically force the rats to creep at higher speeds. The most convenient way of doing this is by the injection of suitably adjusted doses of adrenalin. Qualitatively, the expected effect is produced. In terms of the antecedent analysis it is to be expected that in a broad way the effect of forcing the legs to move more frequently should be entirely similar to that produced by the imposition of an added load. It is to be expected, furthermore, that the threshold slope of surface for the elicitation of definite orientation should be found lower than in the absence of higher speeds of progression, but that at the new threshold slope the *orientation angle* obtained should be statistically identical with that characteristic of the threshold slope of surface which obtains for the untreated rats.

## II

Not only is it true that the orientation angle  $\theta$  as a function of the slope of surface is a characteristic property of each pure line tested, but it has been demonstrated in addition that the *variability* of orientation is also a constitutional property. The scatter of the individual determinations of  $\theta$  at each of a number of slopes of surface, measured

by the respective  $\sigma$ 's, is a rectilinear function of  $\sin \alpha$  for the unweighted, untreated rats; the relative variation of performance ( $\sigma/\theta$ ) is also a rectilinear function of  $\theta$ ; and the quantity which has been termed "the *proportionate modifiable variation*" (Crozier and Pincus, 1931-32*a*, etc.) is likewise a characteristic racial property. The several indices of variability behave in a characteristic manner in individuals produced by cross breeding (Crozier and Pincus, 1931-32*e*, and Paper X).

Since these methods of measuring and expressing variation of performance permit a very precise indication that the observations concerned which satisfy these criteria are free from individual bias or idiosyncrasy due to the observer, and are competent to express an organic invariant of a particular race under the conditions thus far explored, it becomes possible to raise as a fundamental issue the question as to what is really meant by a statement to the effect that a given method of experimental treatment, or an alteration due to a spontaneous or other organically determined constitutional change of an organism, has "increased" or "decreased" this organism's variation of performance. The whole question of the nature and meaning of "variation of response" is indeed at issue (*cf.* Crozier, 1929). The problem is somewhat analogous to that which we have already discussed (Crozier and Pincus, 1927-28, 1929-30*a, b*) in the case of measures of total performance. The fact that for certain of our lines the curves connecting  $\theta$  with  $\alpha$  cross one another shows that it is impossible to characterize the respective geotropic performances except by the complete representation of the creeping curves—no mere measure of maximum performance, for example, is anything but misleading; the behavior of the segments of the respective curves in cross-breeding tests amplifies the ground for this position. So also in the consideration of *variability*; variation of performance must be considered as a function of the performance before a significant index of variability can be obtained. That significant measures are obtainable by this procedure is amply demonstrated by the fact that the indices of variation secured on this basis are constant within narrow limits for each race, but differ pronouncedly for the several races. In practice, then, it will not be adequate to state that under a given set of constant conditions the root-mean-square deviation of the measures of performance has been

increased or decreased with reference to a chosen standard. The behavior of the *total* variation of the performance, and the measurement of that fraction of the total which is modified according to the intensity of excitation, is the necessary basis for any discussion of changes which may be induced in variation of performance. It is easy to show that divergent results of completely contradictory superficial significance may be obtained unless this procedure is followed.

This paper is concerned primarily with these two points: (1) the distortion of the fundamental  $\theta - \alpha$  curve as the result of injection of young rats of standard age with a sufficient dosage of adrenalin chloride to produce an appreciable increase in the speed of progression during geotropically oriented creeping; (2) the modifications thereby induced in the measures of variation of performance. An important additional point which the latter consideration enables us to deal with, is connected with the suspicion (Crozier and Pincus, 1931-32*c*) that the total variation of performance, or the fraction thereof which is a function of the intensity of excitation, may be determined by the total number of effective sensory elements affected through tension in the musculature which supports and moves the animal's mass.

The discussion thus far has been restricted to the consideration of the performance of young rats of standard age (13-14 days after birth). It becomes of very considerable interest to extend these considerations to the treatment of the orientation of *adult* rats. In this case, two possibilities are apparent, and there is no means of deciding beforehand, nor any necessity to decide, which of the two may be expected: either the total number of effective "sensory units" or units of variation may change with age, or these numbers may appear as racial invariants. To this aspect of the problem we shall turn in the succeeding paper.

### III

The present experiments are concerned with young rats of race *A*, 3 individuals (2 males, 1 female) from the 23rd generation of this inbred line with which many previous observations have been made (*cf.* Crozier and Pincus, 1931-32*a*; and subsequent observations). The method of observation has been discussed previously (Crozier and Pincus, 1931-32*a*). These rats received intraperitoneal injections of 2/5

cc. of adrenalin chloride 1:50,000 in sterile Ringer solution before each set of measurements. Control observations, employing injection of Ringer solution only, showed no disturbances whatever of the fundamental  $\theta - \alpha$  relationship to be traceable to the fact of handling and injection. Antiseptic precautions were employed. Beginning 1/2 hour subsequent to the injection and continuing for several hours thereafter, creeping on the inclined surface is "steadier," swifter; creeping is relatively uninterrupted; there are practically no long waits between periods of spontaneous creeping such as are involved in observations on the uninjected, "normal" young rats. Repeating the in-

TABLE I

Mean orientation angles ( $\theta$ ) as a function of slope of surface ( $\alpha$ ) for young rats of strain *A*, injected  $\frac{1}{2}$  hour previously, intraperitoneally, with 2/5 cc. of adrenalin chloride 1:50,000; 3 litter-mates (2 ♂ ♂, 1 ♀);  $n = 40$  observations on each.

Order of measurement	$\alpha$	$\theta$	P.E. <sub>g</sub>
	<i>degrees</i>	<i>degrees</i>	<i>degrees</i>
7	12.5	54.05	$\pm 1.99$
6	15	59.85	1.87
9	20	61.70	1.87
2	25	65.46	1.60
1	30	70.11	1.40
5	40	73.89	1.22
3	50	73.43	1.38
8	60	78.14	0.86
4	70	78.21	0.79

jections after 24 hours leads to an identical succession of events. The sequence of observations was so arranged (Table I) that no unfair influence upon the readings at any one slope was to be looked for.

In Fig. 1 the data of Table I are plotted, as well as the standard curve for uninjected *A* rats; the latter is taken from the material in previous papers, with which sundry subsequent determinations show quantitative agreement. The curve for the injected rats is possibly modifiable at its lower end, but as drawn it passes through the standard deviations of the two lowest points. The effect of the injection with this particular dosage of adrenalin is clearly such that (1) it lowers the threshold slope of surface from approximately  $\alpha = 20^\circ$  to  $\alpha = 12.5^\circ$ .



(2) nevertheless, at this new, lower threshold slope the threshold orientation angle, the threshold response, is statistically identical with that obtained at the threshold slope of surface effective for the uninjected rats; (3) the curve connecting  $\theta$  with  $\alpha$  has been distorted in precisely the same manner as it is (Crozier and Pincus, 1929-30*b*, 1931-32*b*) by the addition of a mass of approximately 2.5 gm. on the saddle region; the lower portion of the curve has been pulled out to the left, the slope has been decreased; this is the effect which we have

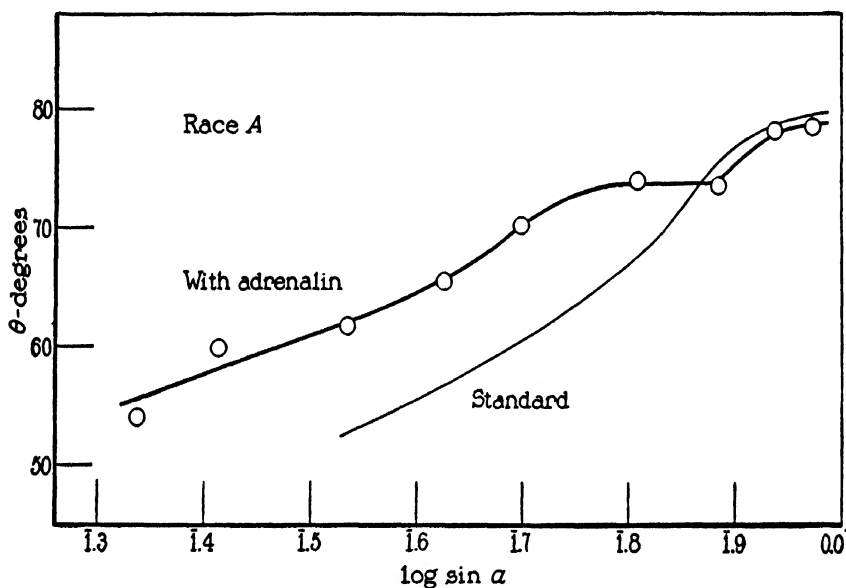


FIG. 1. Modification of the relation between mean orientation angle  $\theta$  and slope of surface ( $\alpha$ ) with young rats of race *A*, by intraperitoneal injection with 2/5 cc. adrenalin chloride 1:50,000. The *standard* curve is derived from many previous measurements with litters of race *A*. The effect is exactly analogous to that obtained by the carrying of an additional load of approximately 2.5 gm. on the saddle of such young rats.

attributed (Crozier and Pincus, 1929-30*b*, 1931-32*b*) to the bringing into action of additional receptor units at slopes of surface much below those characteristically required for their normal activation; (4) this implies a flattened, intermediate region in the curve, as Fig. 1 shows to be in fact demonstrated; and it requires further, in terms of the antecedent analysis, that the uppermost portion of the  $\theta - \alpha$  curve should under these conditions lie *below* the standard curve for un-

treated young rats. Qualitatively, therefore, the effect of adrenalin, involving the increased frequency in the use of the legs during the execution of oriented trails, is precisely that which the assumption underlying our differentiation of the  $\theta - \log \sin \alpha$  graphs (Crozier and Pincus, 1929-30*b*) has implied.

#### IV

The theory of the measurement of *variability* for cases of this type requires that the relative variation of performance should be a rectilinear function of the intensity of excitation. The latter is measured by the orientation angle  $\theta$  (Crozier and Pincus, 1929-30*b*, 1931-32*a*). Fig. 2 shows that for the series of measurements in which adrenalin has been injected, the ratio  $100 \text{ P. E.}_\theta / \theta$  is a declining rectilinear function of  $\theta$ . From this graph, extending from threshold  $\theta$  to maximal  $\theta$ , the following indices are directly computed: (1) the *variation number*,  $V. N._\theta$ ; (2) the *total relative variation*, namely, the area under the graph; (3) and the *proportionate modifiable variation*, namely the fraction of the total area under the graph which is included in the upper, triangular portion. From the earlier experiments with untreated *A* rats we already know that for this race each of these quantities is a statistical constant, at the standard age. The respective values are given in Table II, together with the values obtained from the series of measurements with the adrenalized rats.

The *variation number*,  $V. N._\theta$ , corrected for size of sample in the usual way (Crozier and Pincus, 1931-32*a*, 1931-32*e*), is 3.64 for the adrenalized rats. For the untreated *A* rats (Crozier and Pincus, 1931-32*d*) this index has the value 2.56 in the same units. We have already demonstrated that the magnitude of this index is in no manner disturbed when the young rats are forced to carry added loads (Crozier and Pincus, 1931-32*e*). Fig. 3 shows that with samples of equal statistical weights the estimation of variation of performance, as by a  $\text{P. E.}$ , can give misleading results when the comparison is restricted to a single slope of surface. With the adrenalized individuals the relative variation of performance is more sharply restricted by increasing the intensity of excitation through elevation of the surface on which creeping is allowed to occur. Consideration of the *total variation* of performance, that is, of the area under the variation plot,

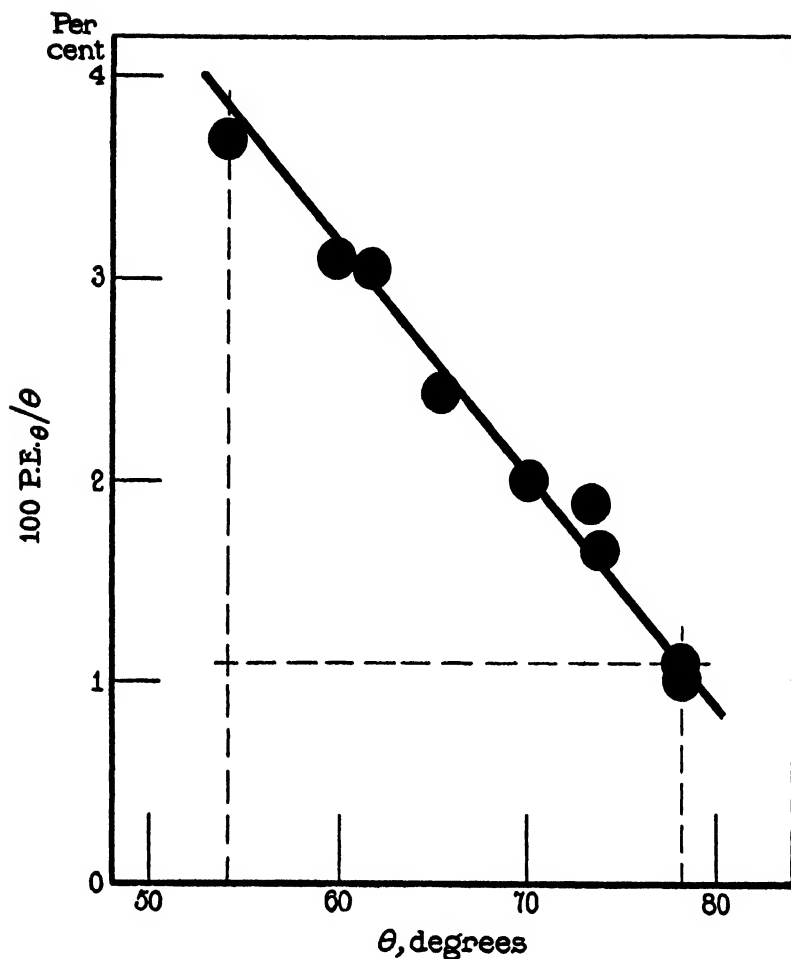


FIG. 2. The relative variation of performance as related to the magnitude of the response, for young rats of race *A* injected with adrenalin. The variation number (*V. N. $\theta$* ) is 3.64, the total relative variation 127 units.

TABLE II

Indices of variation of orientation angles for young rats of race *A*, with and without injection of adrenalin.

Index of variability	<i>A</i> (means)	<i>A</i> with adrenalin
<i>V. N.<math>\theta</math></i> .....	2.56	3.64
Total relative variation.....	84 units	127 units
Proportionate modifiable variation.....	56 per cent	56 per cent

demonstrates that the total variation of performance, between identical sets of limits of intensity of excitation ( $\theta$ ), is markedly enhanced in the case of the adrenalized young rats, namely from 84 units to 127 units. In this respect the action of adrenalin is to be compared with that apparent when genetic influences modify the variation of performance (Crozier and Pincus, 1929-30*a, b*, 1931-32*d, e*). The chance of affecting the total variation of performance, in the present experiments, of course involves variations connected with the process of injection, the absorption of the hormone, the reactivities of the individuals, and the nature of the time course of the action of the adrenalin.<sup>2</sup> In fact, however, the measurements show unexpectedly excellent agreements among the several individuals. Corrected for size of sample and for number of individuals, the total variation ( $100 P. E._g/\theta$ ) *vs.*  $\theta$ , for normal young *A* rats and for young *A* rats carrying additional loads is identical, and amounts to an average of 84.4 units; for the adrenalized young rats in the present series of observations the total variation is 127 units. It is to be concluded, therefore, that the effect of injection with adrenalin, under the conditions stated, gives uniform, reproducible distortion of the  $\theta - \alpha$  curve, and at the same time significantly increases the total variation of  $\theta$  manifest over the entire range of slopes of surface within which geotropic orientation occurs.

The percentage of the total variation which is modifiable as a function of  $\theta$  is completely unaffected, however, by comparison with the findings for the normal young *A* rats. For the latter, with and without additional loads carried, the proportionate modifiable variation ranges from 56 to 59 per cent. For the adrenalized young rats the percentage modifiable variation is still 56 per cent. We have already indicated that there appears to be a connection of some sort between the proportionate modifiable variation of  $\theta$  and the number of sensory units, when diverse races are compared (Crozier and Pincus, 1931-32*c*). It has been shown, also, and the demonstration is amplified in the succeeding paper, that the proportionate modifiable variation appears to be a constitutional property in the sense that it is independent of

<sup>2</sup> It is obvious that the procedures here outlined should permit examination of the existence and nature of *individual* differences in the effects due to experimental treatments.

age of individual. This would be taken to indicate that while the effect of injection with adrenalin has been to increase the relative variation of the measured orientations, this has affected equally those generalized aspects of variation which concern the relative variation of  $\theta$  at all intensities of excitation (the *unmodifiable* variation) and that

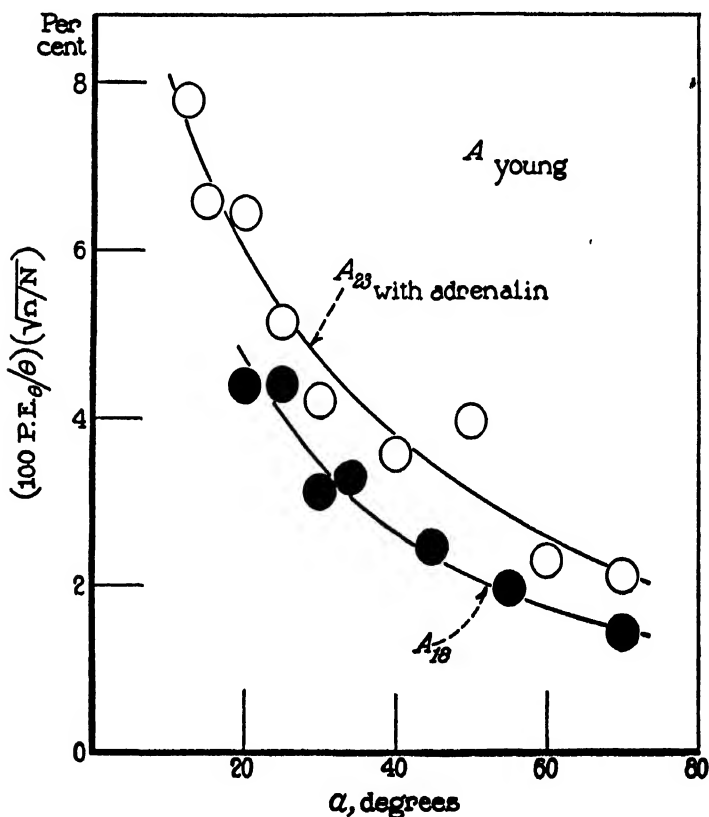


FIG. 3. The relative variation of performance, corrected for the number of observations on each individual ( $n$ ) and for the number of individuals in the set ( $N$ ); data upon a standard litter ( $A_{18}$ , Crozier and Pincus, 1931-32*a*) are taken from an earlier account.

fraction of the total variation which is limited and controlled (*modifiable*) according to the intensity of excitation.

It has been pointed out that the  $\theta - \log \sin \alpha$  curve is distorted, under adrenalin, in a fashion exactly analogous to that evidenced when a weight of approximately 2.5 gm. is attached in the saddle posi-

tion (Crozier and Pincus, 1929-30 *b*, 1931-32 *b*); the differentiation of  $\theta - \log \sin \alpha$  curve shows that the area under the curve obtained by plotting  $(\Delta\theta/\Delta \log \sin \alpha)$  against  $\sin \alpha$  is a little lower than with that for the curve given by the untreated rats; the curve so obtained, giving a picture of the distribution of the tension thresholds for the receptor units involved, is of course entirely similar to that gotten with posteriorly located additional weights of 2.1 gm. (Crozier and Pincus, 1929-30 *b*, 1931-32 *b*), although a little more extreme. The area under this curve cannot be directly compared with other analogous determinations for the *A* race, since the proportionality factor involved in the differentiation is different, and consequently the magnitude of the units on the ordinate scale. The fact that the proportionate modifiable variation of performance remains fixed under these diverse conditions indicates once more (Crozier and Pincus, 1931-32 *b*) that it reflects a fundamental property of the race of individuals considered.

Since the curve connecting  $\theta$  with  $\log \sin \alpha$  for the adrenalin-injected rats is *almost* rectilinear, one may consider  $P. E._\theta/\theta$  as a function of  $\log \sin \alpha$ ;  $V. N._{\log \sin \alpha} = 2.5$ , slightly lower than with the untreated *A*'s (2.82); the percentage modifiable variation computed on this basis is about 52 per cent. We also expect that  $P. E._\theta$  should be nearly a rectilinear function of  $\sin \alpha$  (Crozier and Pincus, 1931-32 *b*) and this is practically the case. At given slope of surface, however, the variation of  $\theta$  is higher with adrenalin (Fig. 3).

# V

It is pertinent to inquire as to the manner in which the effect of the injection of adrenalin could be expected to influence the geotropic orientation. We have been more particularly concerned to obtain an instance illustrating the fashion in which the analysis of variation of performance must be approached, than in deciding upon the nature of the effect of any particular substance when injected. It is clear, however, that the mere fact of increased frequency of stepping could be regarded as bringing into action more frequently, and consequently with increased "mass" effect, an influx of self-generated (proprioceptive) impulses which could be conceived to modify the state of excitability of the central nervous system in a very general way. This could not be traced to a mere increase in the frequency of stepping,

as the experiments with added loads clearly demonstrate the absence of any such effect (Crozier and Pincus, 1931-32 *b*). An influence of the adrenalin upon muscular tonus, such as is actually observable in the slightly different, more flexed, posture of the legs, might be appealed to in such a case. This could be expected to contribute about equally to the possibility of several kinds of deviations from the exactitude of the oriented responses. In a following paper we shall have occasion to point out that with suitably prepared *adult* rats of race *A* this effect of adrenalin is not at all apparent; in this case, the total variation of performance, for a given magnitude of  $\theta$ , is in no respect different from that with untreated *adult A* rats, although the effect of adrenalin upon the  $\theta - \alpha$  curve is in character entirely similar to that which the present experiments with the young rats reveals.

It is difficult to see why the effect of injecting adrenalin should be analogous in all respects (save that involving variation) to the effect of an added mass located posteriorly at the saddle position on the back. One must suspect that the adrenalin acts differentially, under these conditions, upon the excitation of posteriorly located tension receptors, that is, receptors in the posterior pair of legs. Why this should be the case we cannot at the moment decide, and it is immaterial for the present argument.

#### SUMMARY

The intraperitoneal injection of standard young rats of race *A* with 2/5 cc. of adrenalin chloride 1:50,000 results in increased speed of geotropically oriented creeping upon an inclined surface. It was expected that the effect of such increased frequency of stepping must be analogous to that due to imposition of added loads carried by the rats during geotropic progression. This is verified. The curve connecting  $\theta$  with  $\log \sin \alpha$  is distorted, under adrenalin, so as to be comparable to that obtained with an added mass of approximately 2.5 gm. upon the young rat's saddle; the threshold slope of surface for orientation is accordingly lowered, from  $\alpha = 20^\circ$  to  $\alpha = 12.5^\circ$ ; at the new threshold slope of surface the mean orientation angle  $\theta$  is the same as in the absence of adrenalin at the corresponding threshold slope of surface.

The *total variation* of performance is significantly increased in the injected rats, and at given slope of surface the variation is slightly increased. The *proportionate modifiable variation* of response is quite unaffected by the distortion of the  $\theta - \alpha$  curve, and is the same as in standard young *A* rats untreated or carrying additional loads.

It is pointed out that for the consideration of the problem as to whether a given experimental treatment, or a given natural situation, affects in any way the variation of performance of a living system, it is necessary to obtain indices of variability which involve the expression of variation of performance as a function of measured conditions governing the performance.

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# THE RATE OF OXYGEN UTILIZATION BY YEAST AS RELATED TO TEMPERATURE

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## I

The rate of  $O_2$  consumption of a single strain of yeast, *Saccharomyces cerevisiae*, engaged in the metabolic conversion of dextrose, was studied as a function of temperature in the range 3–35°C. An expression of the relationship was sought in the Arrhenius equation:  $k_2/k_1 = e^{\frac{\mu}{R}(\frac{1}{T_1} - \frac{1}{T_2})}$  where  $k_1$  and  $k_2$  are velocity constants (or figures proportional thereto) at the absolute temperatures  $T_1$  and  $T_2$ ;  $e$  is the base of natural logarithms,  $R$  is the gas constant, and  $\mu$ , called by Crozier (1924) for such cases the "temperature characteristic," is a constant with the dimensions of calories per gram molecule.

The constant  $\mu$  was used throughout the experiments as an index of the reproducibility of the relation: rate of uptake of  $O_2$  to temperature. Data upon the constancy of this relation were desired for comparison with temperature characteristics calculated from rates of reduction of cytochrome in the same strain of yeast.<sup>1</sup>

It is realized that in studying such temperature relationships one is probably dealing with the participation of a large number of separate processes in the consumption of oxygen. The measurement, rate of  $O_2$  uptake per unit number of yeast cells, represents the total  $O_2$  consumption of all these metabolic processes. That the total  $O_2$  consumption of all the processes per unit time, when studied as a function of temperature, should satisfy the Arrhenius equation might perhaps seem improbable. It might seem that at constant tempera-

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<sup>1</sup> To be published.

ture the velocity constants of the reactions using  $O_2$  would differ widely among themselves, and also their critical increments, so that no regular linear agreement in the terms of the Arrhenius equation should obtain. Nevertheless, the organization of the diverse cellular processes is such that in some fourteen kinds of related experiments—ranging in diversity from the  $O_2$  consumption of yeast to the  $O_2$  uptake of goldfish—the data do obey the Arrhenius equation with great precision, and moreover give values of the constant  $\mu$  which fall into definite classes for which the extreme values do not depart by more than 5 per cent from the modes.

Crozier (1924, 1924–25) has proposed that in biological systems the constant  $\mu$  may refer to the formation of active molecules or ions of the catalyst controlling the velocity of the slowest process in a catenary or otherwise interrelated series of reactions possessing different critical increments. It is thought that the velocity of the organic activity as a whole is controlled by some substance or by such a dynamically linked system of changes. What actual chemical reactions or units of cellular organization are linked together in this manner, how a control of velocities is established yielding such close agreement of values of  $\mu$ , and correlations with thermal increments obtained in purely chemical reactions, are still subjects of investigation. Perhaps by aid of biochemical procedures such as those developed by Keilin (1929, 1930), Quastel and Wooldridge (1927*a, b*), Harden (1932), and others, it may be possible to gain further information desired for this inquiry.

## II

### *Methods*

The rate of oxygen utilization was measured with Barcroft differential manometers, using cups of 35 ml. capacity having a vertical tube 6 mm. in diameter and 20 mm. high sealed in at the bottom to hold 5 per cent KOH for absorption of carbon dioxide. It is absolutely necessary to increase the surface of the KOH solution by using small rolls of filter paper (*cf.* Keilin, 1929; Dixon and Elliott, 1930), to insure complete absorption of  $CO_2$ . It was found that shaking at a frequency of 120 complete oscillations per minute with a distance of travel of 3 cm. was necessary to obtain reproducible rates of oxygen utilization. Dixon and Elliott (1930) have thoroughly investigated this point.

The calibration constant for each Barcroft differential manometer was obtained

by the method originally described by Hoffman (1913-14), and subsequently simplified by Münzer and Neumann (1917). At constant temperature ( $20^{\circ} \pm 0.01^{\circ}\text{C}.$ ) small volumes of air measured with a calibrated 1 ml. pipette graduated in hundredths were added to the right hand vessel of the manometer. After correcting for the "dead space" between the right hand tap of the manometer and the surface of the mercury in the pipette by a modification of the method of Münzer and Neumann (1917) the calibration constant  $K$  was calculated by the

formula  $K = \frac{v}{p}$ , where  $v$  = volume of air in c.mm., and  $p$  = pressure of the manometer fluid in mm. The right hand vessel was always used for measuring the rate of  $\text{O}_2$  uptake. At the time of calibration each vessel contained 3.0 ml. of phosphate buffer at pH 7.3, 0.3 ml. 5 per cent KOH, and a small roll of filter paper (the volume and area were kept constant for all calibrations and subsequent experiments).

The set-up of apparatus and the procedure of calibrating were essentially the same as those employed by Münzer and Neumann (see their Fig. 3; also, Stephenson, 1930, p. 271).

Münzer and Neumann (1917) showed that  $K$  is independent of the temperature. I tested this point for the same manometer at constant barometric pressure. The values of  $K$  were as follows: at  $20^{\circ}$ ,  $K = 3.94$ ; at  $40^{\circ}$ ,  $K = 4.00$ . The deviation is within the experimental error of determining the values of the constant.

The value of  $K$  varies reciprocally with the barometric pressure (*cf.* Münzer and Neumann, 1917). A correction for this variation was made for each determination. The example given below shows the steps in the calculations.

A correction for the absorption of oxygen at different temperatures was not made since it was found by calculation that such a correction made a difference of only 0.4 per cent in the ratios of rates of  $\text{O}_2$  uptake at temperatures  $10^{\circ}$  apart in the range from  $15$ – $30^{\circ}\text{C}.$  and 0.9 per cent in the range  $4$ – $14^{\circ}$ , and so would not appreciably change the significance of the values of the temperature characteristics obtained.

### *Sample Calculation*

Manometer No. 74.  $K = 3.26$  at **743** mm. Hg barometric pressure at time of calibration.

*Experiment.*—March 11.

Right vessel contained 3 ml. of yeast suspension; 0.3 ml. KOH; roll filter paper.

Left vessel contained 3 ml. phosphate buffer; 0.3 ml. KOH; roll filter paper of exactly the same size and weight as in right vessel.

Temperature was  $35.5^{\circ}\text{C}.$

Barometric pressure was **746** mm. Hg at time of determining  $\text{O}_2$  consumption.

$\therefore$  Vessel constant at **746** mm. = 3.257 (corrected for barometric pressure).

Average rate  $\text{O}_2$  uptake = 51.35 mm. per 10 minutes (*cf.* Fig. 1 for method of calculation).

$\therefore$  Average rate  $O_2$  uptake = 166.73 c.mm. per 10 minutes at 748 mm. Hg by the formula:  $v = Kp$ .

This procedure was followed in calculating the rate of  $O_2$  consumption at each temperature in a series of determinations. Where measurements were made at different barometric pressures, the rates were adjusted to some one barometric

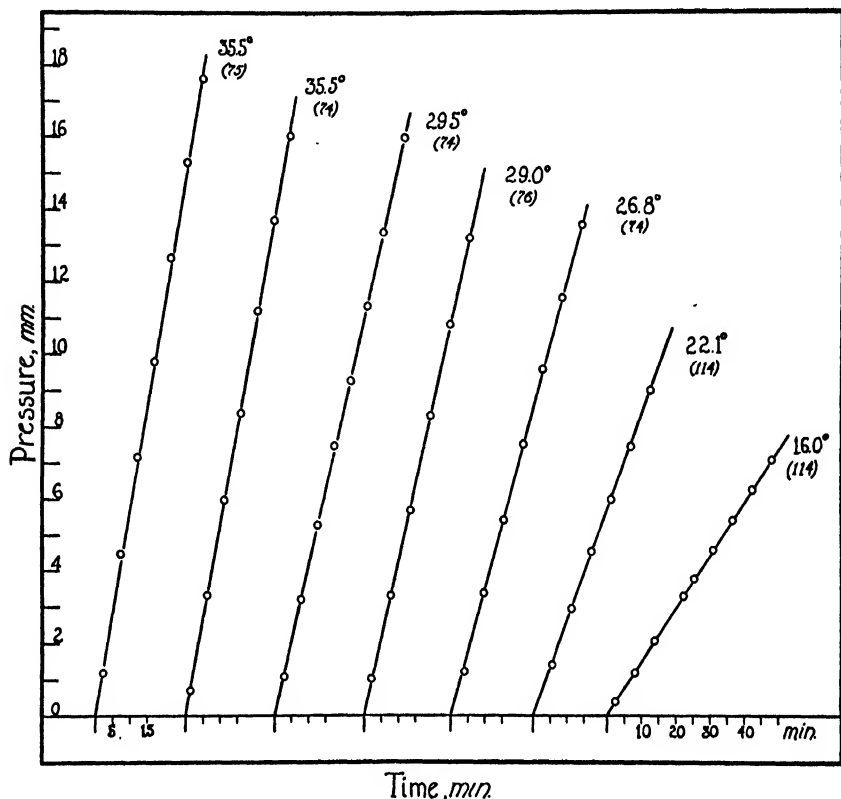


FIG. 1. Oxygen consumption by suspensions of yeast. Ordinate, volume of  $O_2$  taken up, measured as total change in pressure; abscissa, time. Rates of consumption of  $O_2$  were calculated from such graphs. The rate of uptake of  $O_2$  is constant during the period of observation and throughout the range of temperatures employed (3–35°C.). The number placed under each temperature refers to the particular manometer used.

pressure over the whole range of temperatures. In this final form the data were then plotted according to the Arrhenius equation.

A procedure eliminating the use of a calibration constant was also employed for a series of determinations of the temperature characteristics (*cf.* p. 825, Method C). The values of the temperature characteristics as calculated from these relative rates of respiration agree to within 4 per cent with the averages obtained by

the use of absolute rates of  $O_2$  consumption (*cf.* Table I). It is concluded that the simplified procedures used in calibrating the vessels and in calculating the volumes of  $O_2$  consumed per unit of time were sufficiently accurate for the purposes of this investigation.

A method of calculating calibration constants suggested by Dixon (private communication) was also applied to the data obtained in these experiments. The calibration constant  $K$  obtained by the Münzer and Neumann method was first corrected to N.T.P. and dryness at  $20^\circ C$ . (the temperature at which all calibrations were made), yielding a new constant  $K_1$ . Since  $K_1$  varies as  $1/T$  when the volume of the gas dissolved in the liquid in the vessels is negligible compared with the volume in the gas phase (the conditions in these experiments), another constant,  $K_2$ , was calculated for each temperature at which the consumption of  $O_2$  was measured. The rate of  $O_2$  consumption was then obtained by the equation  $v = K_2 p$ .

The average temperature characteristics obtained by these two methods differ only by 1.4 per cent in the range from  $4$ – $15^\circ C$ . and by 1.1 per cent in the range from  $15$ – $30^\circ C$ . The difference between the average rates of respiration calculated by these two methods is also small:—at  $22.8^\circ C$ ., 116.4 c.mm. per 10 minutes by the Münzer and Neumann procedure and 113.3 c.mm. per 10 minutes by the Dixon procedure; at  $7.8^\circ C$ ., 27.8 c.mm. per 10 minutes by the Münzer and Neumann method and 28.33 c.mm. per 10 minutes by Dixon's method. For convenience the Münzer and Neumann method has been used throughout this paper in calculating the rates of consumption of oxygen. The small differences between the values of the temperature characteristics as obtained by the two different methods of calculation do not change the interpretation of the results. For a more critical determination of the magnitudes of  $\mu$ , greater refinement in culturing yeast and greater precision in measuring its rate of respiration are required.

The yeast used was obtained through a local dealer from N. V. Nederlandsche Gist-en Spiritusfabriek, Delft, Holland, and was stated by the company to be "nearly a pure culture as far as this can be obtained by production on a large scale." Indeed, these experiments contain additional proof that, over a period of 6 months, yeast from this source behaved physiologically as a pure culture.

Whenever it was necessary to keep the number of cells constant for experiments made at a series of temperatures on different days, the following procedure was employed: 0.2 to 0.8 gm. of fresh yeast was weighed out and suspended in 100 ml. of  $M/15$  phosphate buffer mixture of pH 7.3. By centrifuging samples of this suspension in graduated Hopkins vaccine tubes it was possible to estimate the number of cells per ml., and then by dilution to keep the relative number of cells per ml. equal throughout all the experiments. This method, however, gives no means of estimating or of adjusting the number of active, respiring cells.

A method of arriving at an index of the volume of "live cells," or better, the level of the rate of oxygen consumption for each suspension, was tried in one series of experiments (*cf.* p. 825). The rate of oxygen uptake of each fresh suspension was determined at the same temperature ( $5^\circ C$ .); the percentage difference from a

selected rate (20 c.mm. per 10 minutes) was then used in correcting the rate obtained at higher temperatures, in the manner illustrated by this example:

Suspension April 12.	Rate at 5.0°C.....	21.5 c.mm. per 10 min.
	Standard rate.....	20.0 " " 10 "
	Difference.....	1.5
	Per cent difference.....	7.5

Rate at 18°C.....	62.0 c.mm. per 10 min.
Corrected rate at 18°C....	57.35 " " 10 "

In this manner the rates were adjusted for all the temperatures in the series (*cf.* line *B* of Fig. 3).

This method has limited applicability since it can be used only when the supply of yeast is produced under identical conditions of culture throughout the entire period of experimentation. Warburg (1927) found that the same strain of yeast cultured in different media or under different conditions yielded values of  $Q_{O_2}$  (c.mm. of  $O_2$  per hour per mg. dry weight) which differed widely. The yeast used in these experiments was produced under identical conditions throughout the entire period of the investigation.

### III

#### *Determination of Temperature Characteristics*

##### *Method A*

0.8 gm. of yeast was suspended in 100 ml. of buffer solution at pH 7.3. The suspension was kept in an ice bath at 1–2°C. Sufficient dextrose to make a 1 per cent solution was added to 25 ml. of the suspension 1 hour before beginning the determination of its rate of oxygen uptake; during this period oxygen was constantly bubbled through the suspension, which was kept in the ice bath. The cups of the manometers were rapidly prepared, with 3 ml. of the suspension in each right cup and 3 ml. of buffer mixture in each left cup. The manometers were immediately placed in the thermostat and shaken at the rate of 120 oscillations per minute. After 15 minutes had elapsed for temperature equilibrium, the stop-cocks were closed and readings taken every 8 or 10 minutes.

The constancy of pH of the yeast suspensions was ascertained at different temperatures with Clark-Lubs indicators. This method is sufficiently accurate for the purpose. (Data of Hastings and Sendroy

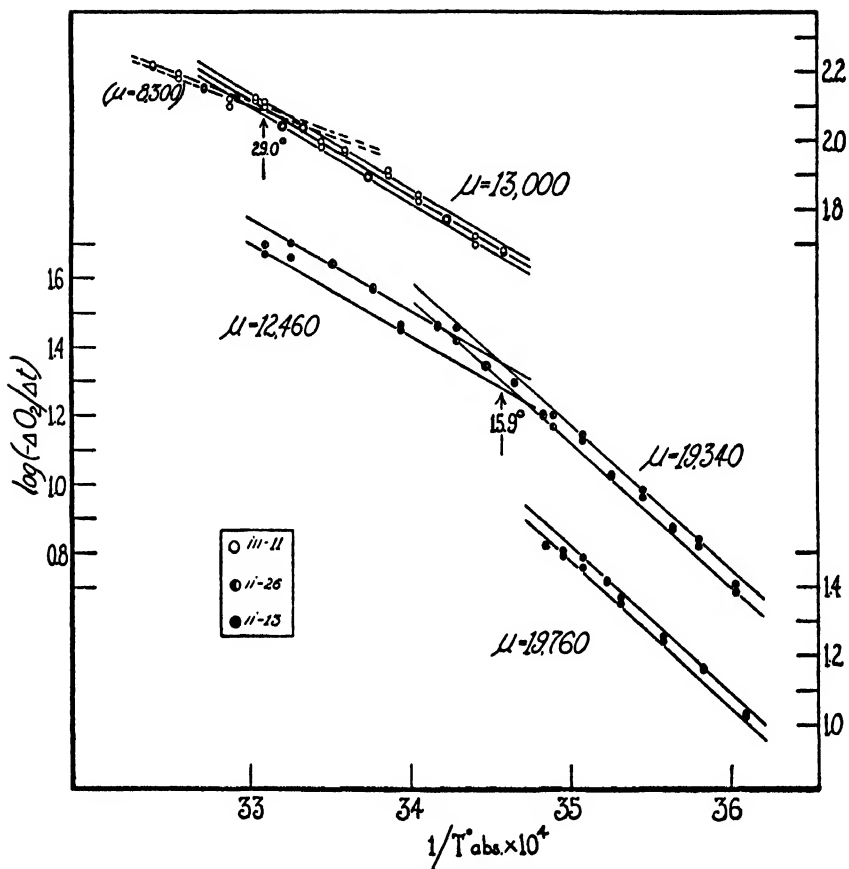


FIG. 2. Data on the relation of rate of  $O_2$  consumption to temperature are plotted according to the Arrhenius equation;  $\mu$ , called the *temperature characteristic*, computed from the slope of the graph, is a constant having the dimensions calories per gram molecule.

These three sets of data resulted from experiments made on three suspensions of yeast. Each run, lasting not more than 9 hours, was made on the same day. In each experiment the thermometric scale was first ascended by intervals of  $2-3^\circ$ . Observations at alternating temperatures were made by descending the temperature scale in similar steps. Thus any change in the rate of respiration of the stock suspension (which was kept at  $1-2^\circ C.$ ) could be detected in the scatter of the points on the arith-log plot. (See also text for further comments on the constancy of the rate of uptake of  $O_2$  by the stock suspension.)

Each point is an average of at least four rates, obtained as shown in Fig. 1, for separate samples of a stock suspension.

Above  $30^\circ$  the data are too few to give great weight to the value of  $\mu$ ; the determinations are chiefly useful in locating the critical temperature at  $29^\circ$ .

The suspension dated ii-13 contained 0.007 gm. of yeast (wet weight) per ml.; ii-26, 0.007 gm. per ml.; iii-11, 0.008 gm. per ml.

Calibration constants obtained by the Münzer and Neumann procedure.



(cf. Clark, 1928) show that a M/15 phosphate mixture of pH 7.3 changes only 0.02 pH when the temperature is raised from 20° to 38°C.)

For suspensions containing 0.002 gm. of yeast per ml. and 1 per cent dextrose, it was found that the pH remained constant at 7.3 for 3 hours at 16.4° (the rate of O<sub>2</sub> uptake remaining constant for the same period of time), and for 2 hours at 35°. A suspension containing only 0.004 gm. per ml. remained constant for 1 hour at 35°; and for 30 hours at 1–2°C. Since the determinations of rate of O<sub>2</sub> consumption were made in 30 minutes at 35°, in 50 minutes at 16°, it can be assumed that the data were all obtained at constant H ion concentration.

The temperature of the thermostats was held constant, varying, on the average, by less than  $\pm 0.1^\circ\text{C}$ . Above room temperature the cooling of the thermostat was exactly balanced by adding heat from a long carbon filament lamp whose depth of immersion was adjustable and whose heat could be regulated by a hand-operated rheostat. Below room temperature a special cooling unit was devised (Stier, 1931) as a substitute for the more expensive SO<sub>2</sub> compression circuit (Crozier and Stier, 1926–27*b*) which can be used for low temperature thermostats. This device consisted of a copper funnel whose spout was closed by a rubber stopper and had a hopper of heavy linoleum attached to its top. The hopper and funnel were filled with pieces of cracked ice about the size of a walnut. Water from the melting ice was removed by a suction line attached to a water aspirator. The unit was attached to a ring-stand by a clamp which allowed the conical portion of the funnel to be lowered into the water bath to various levels. In operating the device, a depth of immersion is found, by trial, where the heat removed from the bath is about equal to the heat added from the surroundings.

In the absence of a system of automatic thermoregulation a position of balance was maintained by frequently reading the temperature on a calibrated thermometer (about once every 4 minutes) and then adjusting the depth of immersion of the cooling unit in the thermostat. Temperatures as low as 3° when the room temperature was 16° were maintained to within  $\pm 0.05^\circ\text{C}$ . With automatic thermoregulation, this cooling unit (with certain modifications) has given a constancy of temperature regulation within  $\pm 0.007^\circ\text{C}$ . (Stier, 1931).

By using 4 thermostats it was possible in one series of determinations to make 32 measurements of rates of O<sub>2</sub> uptake at 16 temperatures in 9½ hours over the range 16–35°; in another series of experiments 34 determinations were made in 9 hours at 17 different temperatures over the range 4–30°C.<sup>2</sup> In these two sets of experiments I was assisted

<sup>2</sup> See legend of Fig. 2 for comments on the constancy of the rate of oxygen uptake of the suspension during the 9 hour period of experimentation.

in making observations by Professor Barcroft, Dr. G. Enders, and Dr. P. Rothschild, to whom I am deeply indebted for this help.

Experiments on single suspensions of yeast are recorded in Fig. 2. The average values of the temperature characteristics ( $\mu$ ) for two

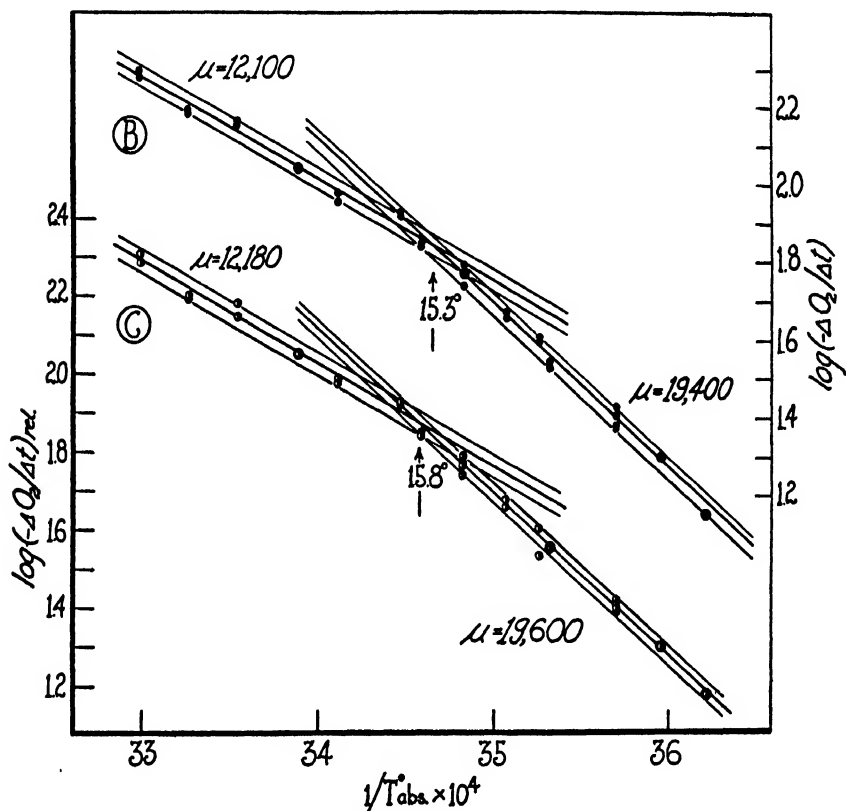


FIG. 3. The data plotted on lines B and C were obtained on different days, a new suspension of yeast being made up for each determination. The methods of correcting the rates of  $O_2$  uptake for variation in the number of cells in different suspensions are explained in the text.

Each suspension contained 0.016 gm. (wet weight) of yeast per ml. of buffer mixture.

Calibration constants calculated by the Münzer and Neumann procedure.

series of experiments (February 26 and March 11) were found to be:  $35-30^\circ$ ,  $\mu = 8,290$  calories;  $30-15^\circ$ ,  $\mu = 12,730 \pm 270$ ;  $15-3^\circ$ ,  $\mu = 19,340$ . In a shorter series covering the range  $15-3^\circ$ , the value of  $\mu$  was found to be 19,760 calories.

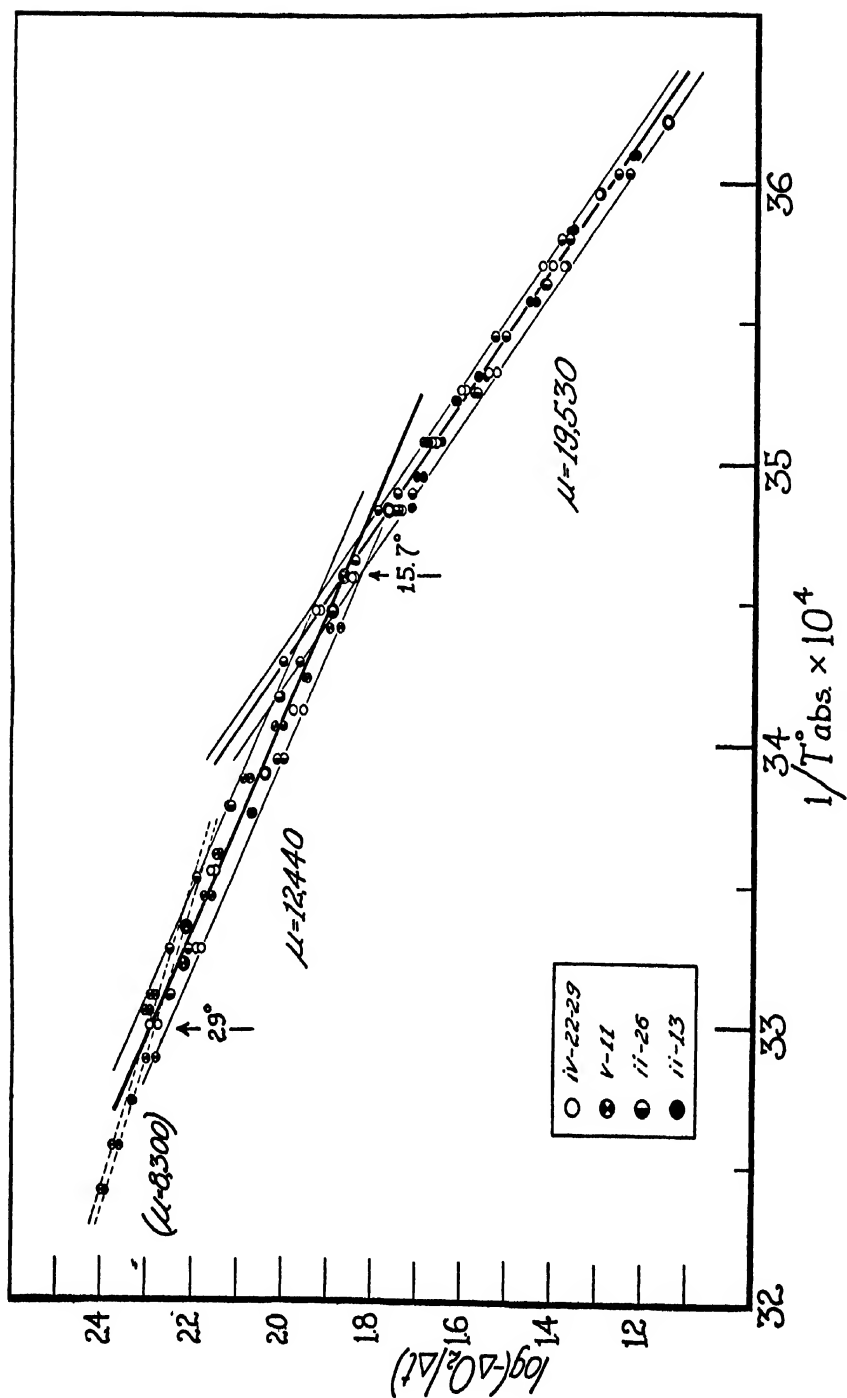


FIG. 4

### Method B

The values of the temperature characteristics secured by Method A were compared with values obtained by a procedure in which a fresh suspension was employed at each temperature. To correct for any variation in the total number of "live cells" in new suspensions made up on different days the method of adjustment described on p. 820 was used.

Line B of Fig. 3 gives the data obtained by this procedure, the logarithms of the rates being plotted against the reciprocals of the absolute temperatures according to the Arrhenius equation. The graph is similar to that obtained by the previous method of experimentation. The points lie on two intersecting straight lines, the values of the temperature characteristic being:  $30-15^{\circ}$ ,  $\mu = 12,100$ ; and  $15-3^{\circ}$ ,  $\mu = 19,400$ .

### Method C

Method C consisted in calculating ratios of  $O_2$  uptake for the temperature range  $3-30^{\circ}$ , thereby endeavoring to eliminate such errors as might have entered the data during their conversion from readings of pressure in mm. of manometer fluid to rates of  $O_2$  uptake in c.mm. at normal pressure (or at some one pressure for the whole range of temperatures). These ratios were obtained for the same manometer by dividing the pressure difference between the two arms of the manometer, per unit time at  $5^{\circ}$ , into the mm. difference in pressure appearing per unit of time at each of thirteen different temperatures. When these data were plotted according to the Arrhenius equation (line C of Fig. 3) the points were fitted by two straight lines intersecting at  $15.8^{\circ}C$ :  $30-15^{\circ}$ ,  $\mu = 12,180$ ; and  $15-3^{\circ}$ ,  $\mu = 19,600$ .

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FIG. 4. Mass plot of the data presented in Figs. 2 and 3. The rates of  $O_2$  consumption from the different experiments are brought together with data in the series marked "iv-22-29" by multiplying each average rate of  $O_2$  uptake in a series of determinations by a factor; for series ii-13, factor taken at  $8.6^{\circ} = 1.677$ ; ii-26, factor taken at  $11^{\circ} = 1.774$ ; iii-11, factor taken at  $22.8^{\circ} = 1.503$ .

$O_2$  consumption, c.mm. per 10 minutes, is given in Fig. 6.

By Dixon's method of calculating calibration constants (*cf.* p. 819) the average values of the temperature characteristics for the data presented in Figs. 2 and 3 were found to be:  $35-30^{\circ}$ ,  $\mu = 8,150$  calories;  $30-15^{\circ}$ ,  $\mu = 12,300$ ;  $15-3^{\circ}$ ,  $\mu = 19,250$ .

In these experiments 5° was used as the reference temperature. Crozier (1925-26) pointed out that this temperature occurs in a critical zone where irregularities often appear in the relations between rates of vital processes and temperature. The procedure used in Methods B and C, may therefore be open to the criticism that the observed rate of respiration at the reference temperature 5° may be "abnormally" high or low, or irregular. All the facts in the experiments, however, are in accord with the data obtained by Method A: (1) *cf.* especially Fig. 4, where the data obtained by Methods A and B are brought together; (2) *cf.* Fig. 2, where determinations of rate of O<sub>2</sub> uptake for temperatures below 5° fall well on the line; (3) note that

TABLE I  
*Temperature Characteristics ( $\mu$ ) Obtained by Different Methods*

Method	$\mu$	Critical temperature	$\mu$	Critical temperature	$\mu$
A (Feb. 13) (Feb. 26) (Mar. 11)	8,290	29.0°	12,460	15.90°	19,760
			13,000		19,340
B			12,100	15.25°	19,400
C			12,180	15.84°	19,600
Average . . . . .	8,290	29.0°	12,440	15.66°	19,530

the average temperature characteristics obtained by Methods B and C agree to within 5 per cent with values obtained by Method A in the range 30-15°; to within 0.25 per cent in the range 15-3°. It appears, therefore, that the use of 5° as a reference temperature has not introduced any large error into these particular determinations. This matter will be thoroughly tested by future experiments.

#### IV

#### DISCUSSION

##### *a*

In the published accounts of respiratory processes of yeast and bacteria there are few data on rates of reactions studied carefully enough

at an adequate number of temperatures so that the measurements can be used for the kind of analysis desired. Slator's data (1906, 1908) on CO<sub>2</sub> production by yeast during fermentation are the only measurements made over a wide enough range of temperatures to be of any use for a temperature analysis.<sup>3</sup> Crozier (1924-25) calculated temperature characteristics for these data and found that the values of  $\mu$  were within the range of critical increments obtained for other sets of data on rates of respiration. The temperature characteristics are: for brewery, distillery, and wine yeasts, 40-22.5°,  $\mu = 12,250$ ; and 22.5-5°,  $\mu = 22,200$ . These are the same data used by Fulmer and Buchanan (1928-29) as the basis of their criticism of the curve fitting employed in this method of temperature analysis (*cf.* p. 832). I have recalculated values of  $\mu$  for these data and have also calculated temperature characteristics for measurements of the CO<sub>2</sub> production by brewer's yeast fermenting various sugars (Slator, 1908). These values have been recorded in Table II. The wide scatter of the observations on the Arrhenius plot makes the significance of the calculated values of  $\mu$  very doubtful (*cf.* footnote 3); the values are given only because of the historical interest of the original measurements. CO<sub>2</sub> production by brewery, distillery, and wine yeasts yields values of  $\mu$  varying from 12,200 to 13,600 for the range 35-20°; and 22,200 to 25,200 from 20-5°. The "break" occurs at about 20.2°C. For CO<sub>2</sub> production by brewery yeast plus dextrose, or with levulose:  $\mu = 12,200$  to 12,900, 35-20°; and  $\mu = 25,300$ , 20-5°; "break" at 20.2°. For brewery yeast plus maltose:  $\mu = 12,100$ , 40-23°; and 32,200, 23-10°; "break" at 22.8°. (*Cf.* Table II for further comments on these values.)

The temperature coefficient ( $Q_{10}$ ) has been employed extensively in the past to describe the "effects of temperature" on various activities of organisms. Although devoid of any theoretical significance, it has been used also in attempts to interpret the underlying chemical

<sup>3</sup> Unfortunately the data are given only in the form of ratios calculated for adjoining temperatures 5° apart, from rates of CO<sub>2</sub> production which did not remain constant during each experiment (see Slator's sample calculation, 1906, p. 136). No mention was made of the constancy of the pH, or of the constancy of the temperature. Consequently calculations of temperature characteristics, or estimations of critical temperatures are without much significance.

mechanisms of vital processes. The value of  $Q_{10}$  is not constant over a given temperature range; its magnitude varies as a function of temperature. As a means of classifying vital processes by their controlling reactions, basing the analysis upon correlations with physicochemical data, the "temperature coefficient" is valueless. A consideration of the disposition of the data in the Arrhenius plot on two or three intersecting straight lines makes clear the fact that the value of  $Q_{10}$  depends upon the  $10^\circ$  span on the temperature scale selected for the ratio.

Crozier (1924-25, 1925-26*b*) has calculated from published accounts the value of  $\mu$  for the relation of temperature to the velocities of various vital processes. The frequency distribution of these temperature characteristics as calculated from practically all known series of observations exhibits a number of discrete modes at: 8, 11, 12, 16, 18, 20, 22, 24, 32 thousand calories. There is evidence that the values 11,000 and 12,000 are organically distinct; similar considerations hold for the values 16,000 and 18,000. For living processes in which rates of  $\text{CO}_2$  production and  $\text{O}_2$  consumption have been measured, the most frequently occurring values of  $\mu$  have been 11,500 and 16,000. In this connection it is striking that data in the literature on reactions whose rates are controlled by the oxidation  $\text{Fe}^{++}$  to  $\text{Fe}^{+++}$ ; respiration of echinoderm eggs with which the theory of catalysis by Fe is intimately connected (Warburg and Meyerhof, 1913; Warburg, 1914); and the deoxygenation of hemoglobin (by CO) (Hartridge and Roughton, 1923*a, b*)—in all these cases the temperature characteristic was found to be  $\mu = 16,300 \pm 200$  calories. The value  $\mu = 11,500$  has been obtained by Rice (1923) for cases where the free hydroxyl ion seems to be the controlling catalyst, and the value  $\mu = 19,000$  to 20,000 for reactions where the unhydrated H ion is the governing catalyst. Rice predicted that chemical reactions should fall into comparatively few classes, each class having its characteristic thermal increment, where  $\mu$  refers to the controlling catalyst of that group. Crozier (1924, 1925-26*b*) found this to be suggestively valid for vital processes, since the values of  $\mu$  could be placed in a small number of classes (*cf.* also Table II).

In Table II I have summarized temperature characteristics for  $\text{O}_2$  consumption and for  $\text{CO}_2$  production calculated by Crozier and others from measurements already published. From a review of this table

it appears that there is a system of interrelated chemical reactions involved in respiration in living matter of diverse kinds (*cf.* Crozier, 1925-26). Processes as different as the frequency of "activity periods" of organisms (Stier, 1930) and the frequency of heart beat (Crozier and Stier, 1926-27*b*) are under the control of this nexus of processes. There are a number of additional unpublished cases which substantiate this view. Another line of evidence also points to a uniform organization of respiratory systems in diverse kinds of living matter. Investigations by Keilin (1925), Shibata and Tamiya (1930), and others show that the respiratory pigments, especially cytochrome, are widely distributed among organisms (from yeast to mammals), participating as key units in processes of cellular respiration.

Table II shows that the temperature characteristics for O<sub>2</sub> uptake of yeast—8,290; 12,440; 19,530—as reported in this paper, have also been found for respiratory processes in other organisms. This series of temperature characteristics is obtained for (respiratory) pulsations of the cloaca in certain holothurians: in *Holothuria captiva*,  $\mu = 20,500$  (Crozier, 1915, 1916); in *Stichopus*,  $\mu = 12,200$  (Crozier, 1916); in *Thyone briareus*, aboral end intact,  $\mu = 12,300$ , and for the isolated aboral end,  $\mu = 8,500$  (Crozier and Stier, unpublished).

The "biological reality" of temperature characteristics is best illustrated by a series of careful experiments on the relation of the frequency of breathing movements to temperature recently reported by Pincus (1930-31). Young mice of a selected line of a *dilute brown* strain of mice and mice of the same age of an inbred *albino* strain gave certain definite values of  $\mu$  and certain definite critical temperatures.

"The F<sub>1</sub> hybrids of these 2 strains, and the backcross generations to either parent strain, exhibit only those 4 values of the temperature characteristic observed in the parent strains and none other. One may therefore speak of the inheritance of the value of the constant  $\mu$ . Furthermore, there appears to be inherited the occurrence (or absence) of a critical temperature at 20°C. The results of these experiments indicate that the particular values here observed have reality in a biological sense. For one can conceive of no more rigid test of the biological integrity of a process than that its descriptive constants are recoverable in successive generations of crossbreeding (*cf.* Pincus and Crozier, 1929; Crozier, Stier, and Pincus, 1929)" [Pincus, 1930-31, p. 442].



TABLE II  
*Temperature Characteristics for Consumption of O<sub>2</sub> and Production of CO<sub>2</sub>*

Yeast	Object	Observer	Classes of $\mu$ , in thousands					
			8	11	12	16	20	30
O <sub>2</sub> consumption, baker's yeast . . . . .		Kubowitz, 1929 ( <i>cf.</i> p. 477)			(14,700?)		(21,970?)	
CO <sub>2</sub> production { brewery yeast distillery yeast wine yeast		Slator, 1906			{ (12,200 to 13,600)		(22,200 to 25,200)	
CO <sub>2</sub> production { brewery yeast + levulose brewery yeast + dextrose brewery yeast + maltose		Slator, 1908			{ (12,200 to 12,900) (12,100)		(25,300)	32,200
Aerobic glycolysis, baker's yeast		Kubowitz, 1929				(16,360?)	(25,420?)	
Anaerobic glycolysis, baker's yeast		Kubowitz, 1929					(25,086?)	
Velocity of oxidation of glucose		Aberson, 1903			(13,420?)			
Growth: rate multiplication. "Time to produce a standard crop"		Richards, 1928	8,600			16,000		
<p>The following distribution of temperature characteristics has been made from Crozier's tabulation (1924-25) of values of <math>\mu</math> calculated by him for the majority of published experiments of O<sub>2</sub> consumption and CO<sub>2</sub> production. The original references can be found in Crozier's paper (1924-25, p. 200). New values entered by the writer have been noted.</p>								
<i>Oxygen consumption</i>				11,800		16,140		
				11,500		16,700		
						16,800	22,000	
						16,850		28,000
						16,200		

<i>Oxygen consumption</i>	
11,500.....	29,500
11,000.....	16,300
	16,000
11,500	
	16,500
	16,100
	16,800
	21,000
11,700.....	16,600*
13,100.....	21,050†
	19,300‡
<i>Carbon dioxide production</i>	
8,100§.....	16,700
	16,200
	12,400
	16,250
	16,200
11,500.....	29,500
	22,000
	16,500¶
	16,100.....
	24,000**
	20,750††
8,290±	12,440±
	19,530±
Average temperature characteristics for O <sub>2</sub> uptake of yeast as calculated from data by Methods A and B.....	

\* O<sub>2</sub> consumption of *Lupinus albus* (Tang, 1930-31). † O<sub>2</sub> consumption of *Zea mays* (Tang, 1930-31).

‡ O<sub>2</sub> consumption of *Azotobacter vinelandii* (Lineweaver, Burk, and Horner, 1931-32). § Added from data of Kuyper, calculated by Navez (1928-29); its companion value, 16,200, has already been reported by Crozier (1924-25, p. 200).

|| CO<sub>2</sub> production of seedlings of *Vicia faba* (Navez, 1928-29). ¶ CO<sub>2</sub> production of seedlings of *Phaseolus aureus* Roxb. (Crozier and Navez, 1930-31).

\*\* CO<sub>2</sub> production of *Lupinus albus* (Tang, 1931-32). †† CO<sub>2</sub> production of *Zea mays* (Tang, 1931-32).

## b

Recently, Bělehrádek (1930), and Fulmer and Buchanan (1928-29), have criticized the method employed by Crozier and others of fitting two or three intersecting straight lines to data plotted according to the Arrhenius equation. It has been objected by these authors that the graph should be constructed as a smooth curve. (In making these criticisms they have omitted a large number of pertinent observations supporting the method of treating temperature data.) A method of testing the validity of fitting intersecting straight lines to the data is illustrated by Figs. 5 and 6, where the rates of  $O_2$  uptake are plotted against  $^{\circ}C$ . The plotted points are the actual observations; the outer edges of the cusps were carried over from the extreme edges of the

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*Footnote to Table II.*—A dotted line connecting two increments means that a "break" has occurred, giving two temperature characteristics, one for the lower range of temperatures, the other for the upper range.

The temperature characteristics for  $CO_2$  production also appear in Table II under the heading " $O_2$  consumption." The same increments are obtained for both processes (*cf.* Crozier, 1924-25, experiments of Brunow, and von Buddenbrock and von Rohr).

Recent experiments by Cook (1930) on "a comparison of the dehydrogenation produced by *B. coli* in the presence of  $O_2$  and methylene blue" when the organism has been treated with toluol according to the procedure of Quastel and Wooldridge (1927*a* and *b*) give points on the Arrhenius plot which are not rectilinear when replotted upon a fair scale. The best straight line is obtained when lactate is dehydrogenated in the presence of methylene blue. These observations give  $\mu = 15,300$  according to my calculations. I have also calculated rates of  $O_2$  uptake from his data—c.mm.  $O_2$  uptake after 15, 30, 45, and 60 minutes—and find that these rates are not constant over the whole temperature range, 40-15 $^{\circ}C$ . The use of inconstant rates is sufficient to account for the curvilinear  $\mu$  plots obtained for  $O_2$  uptake under his experimental conditions; in any case, no analysis can be attempted when the basic velocity is a function of time as well as of temperature (*cf.* Tang, 1930-31; Crozier and Navez, 1930-31). It has been pointed out by Tamiya and Tanaka (1930), and von Euler and Hellström (1930) that upon the addition of toluol to an organism there is a gradual denaturing of the cytochrome system and so a gradually increasing inability on the part of the organism to utilize molecular oxygen. It is obvious that the temperature analysis can be used only when the rate of performance is constant, unless—with a system in which the measured rate is changing in a simple manner—it becomes possible to compute an initial rate undistorted by the progress of changes which are a function of time.

parallel bands enclosing the observations in the mass plot (Fig. 4) in the ranges of temperature: 35–30°; 30–15°; 15–3°. It is preferable to enclose these determinations within three such fan-shaped areas cutting across each other at 15° and 30°—"one" curve cannot fit the data. A full discussion of these matters can be found in: Crozier and Fede-

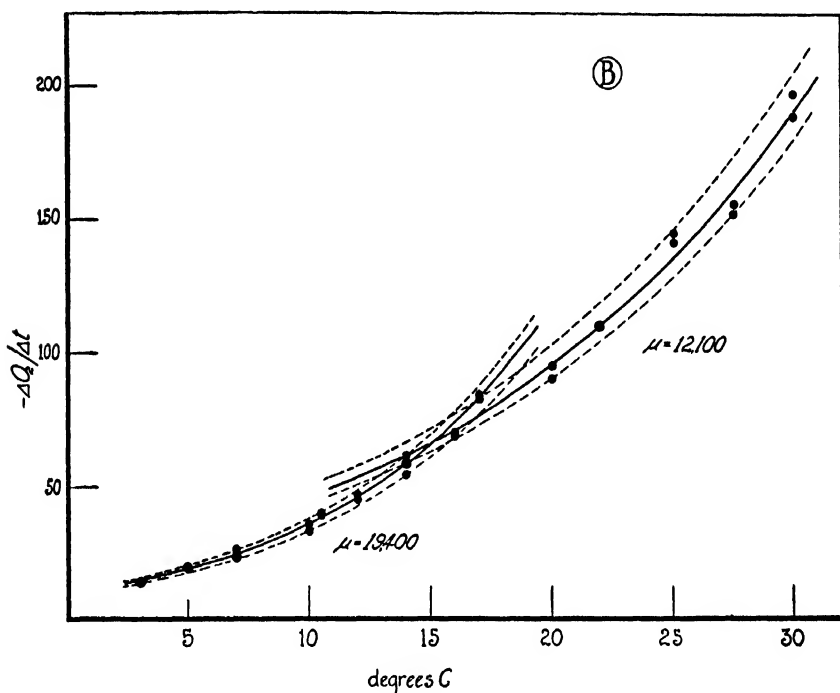


FIG. 5. The rates of uptake of  $O_2$  are plotted in Figs. 5 and 6 as ordinates, and °C. as abscissae. The boundary lines marking the latitude of variation in the Arrhenius plot have been directly carried over from Figs. 3 and 4 to Figs. 5 and 6. Figs. 5 and 6 make it obvious that *one* smooth curve cannot be drawn through the data over the entire temperature range. In Fig. 5 the points are the actual values of rates of  $O_2$  uptake already plotted (in Fig. 3, line B) according to the Arrhenius equation. Fig. 6 is a mass plot of all the data used in Fig. 4.

righi (1924–25); Crozier and Stier (1926–27*a*); Brown (1926–27); Navez (1930).

Attention is called to the significance of the fan-shaped enclosures seen in Figs. 5 and 6. Such a distribution of points means that the extreme limits in each fan increase with temperature; *i.e.*, for each range 3–15°, 15–30°, and 30–35°. This kind of spread of the observa-

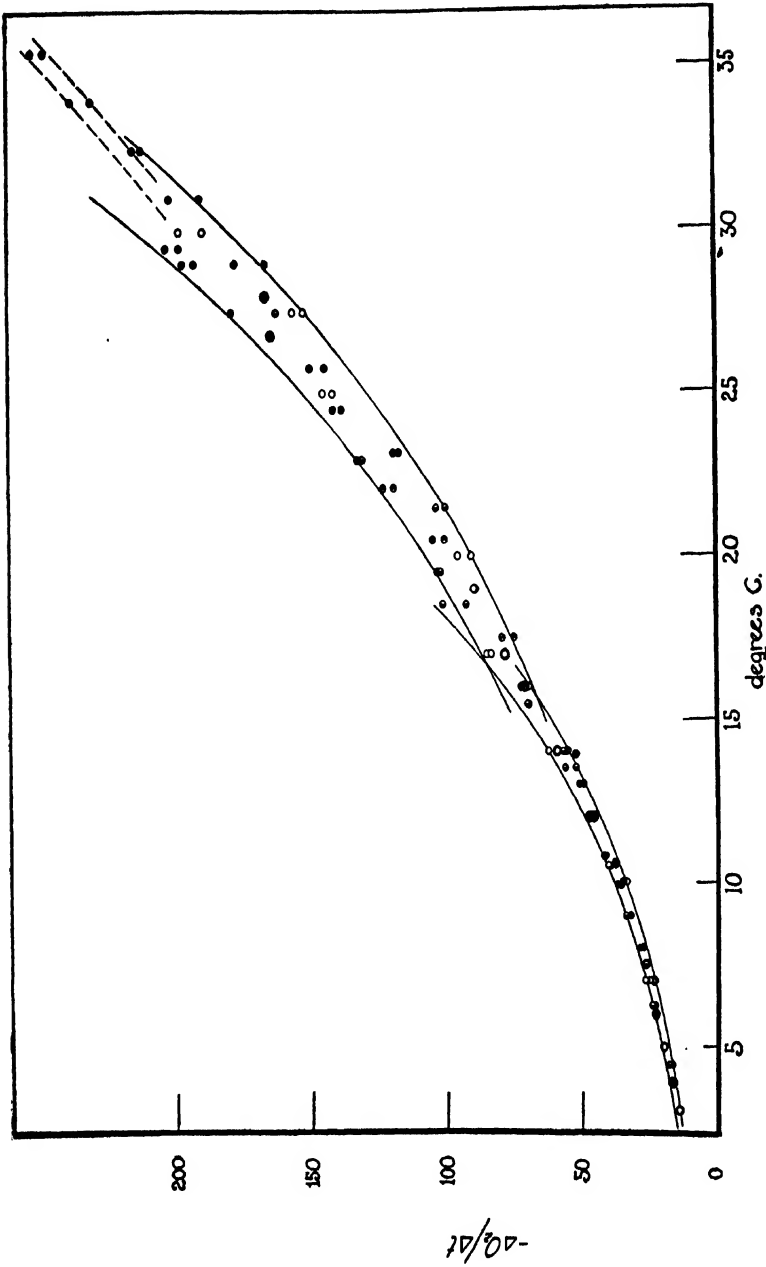


FIG. 6. See legend of Fig. 5.  
The symbols used in Fig. 6 refer to the same series of experiments as the symbols in Fig. 4.  
Calibration constants calculated by the Münzer and Neumann procedure.

tions cannot be due entirely to experimental errors, since such errors would certainly be a constant amount for each temperature and the percentage differences from the average would then alter with temperature. A constant percentage difference gives parallel sets of lines in the Arrhenius plot. This result has been obtained typically where rates of vital phenomena have been studied as a function of temperature (*cf.* papers by Crozier and associates, *J. Gen. Physiol.*, 1924, to date).

The actual extreme percentage differences at each temperature as calculated from the mass plot (Fig. 4) are found to be constant within each range of temperature:  $3-15^{\circ}$ ,  $\pm 6.1$  per cent of the mean;  $15-30^{\circ}$ ,  $\pm 8.5$  per cent;  $30-35^{\circ}$ ,  $\pm 1.8$  per cent. The latitude of variation changes at a critical temperature, being greatest in the range  $15-30^{\circ}$  and least in the range  $30-35^{\circ}$ . A part of this latitude of variation is of course spurious, being due to the fact that the variation in different series of measurements is not quite the same; thus, to give one illustration, data which appear in Fig. 2 (iii-11) exhibit in fact a maximum latitude of variation of  $\pm 5$  per cent over the range  $15-30^{\circ}$ .

A similar case in which an increase in the latitude of variation accompanies a change in the value of the temperature characteristic is found in the experiments on speed of gliding motion of *Beggiatoa* (Crozier and Stier, 1926-27*a*).

When the logarithm of the probable error of the mean rate of such a vital process at each temperature is plotted according to the Arrhenius equation the same  $\mu$  is obtained as was found for the mean rates of the process as a whole (Crozier, 1929; Navez, 1930; Pincus, 1930-31), when  $n$  is constant, *i.e.*, the latitude of variation of the observations is a constant percentage of the mean, and both mean and variation of the mean hold the same relationship to temperature. This has been interpreted to mean that the utilization of  $O_2$  at constant temperature, let us say, occurs at a rate which varies not only within the error, but goes through a cyclical variation such that the extreme limits fall on lines which are a constant percentage of the mean throughout the specific range of temperatures. A cyclical fluctuation in frequency of respiratory movements of grasshoppers has already been found (Crozier and Stier, 1925-26). Whether a similar minor cyclical variation in the respiratory activity of suspensions of yeast cells exists will be tested by subsequent experiments.

## C

Crozier (1925-26) has designated by the name "critical temperature" a temperature in the Arrhenius plot at which two lines intersect, or a temperature above or below which "destructive" changes occur in the organism, such that the rates measured become a function also of *time*. Critical temperatures were obtained from numerous published accounts of experiments on the relation of temperature to rates of vital processes, and were made into a frequency polygon. The distribution was found to be multimodal, with the greatest number of cases falling in the 15° class. Other modes were at 4.5°, 9°, 20°, 25°, 27°, 30°. Subsequent data (*cf.* papers by Crozier and associates, *J. Gen. Physiol.*, 1925, to date) have confirmed this series of critical temperatures.

The following classes of critical temperatures have been obtained from the published accounts of experiments on O<sub>2</sub> uptake and CO<sub>2</sub> production previously quoted in Table II.

Classes of critical temperatures .....	9°C.	15°C.	17°C.	20°C.	25°C.	27°C.	30°C.
Instances	9.0	14.0 15.5 15.0 15.0 15.2 15.2	17.0 17.0 17.0 (18.2)	20.2 22.7 19.5 19.5 21.0 21.0 20.0 20.0	25.0	27.0*	30.0 30.0 30.0† 30.0

The average critical temperatures here found for O<sub>2</sub> uptake of yeast are:

$$15.7^{\circ} \pm 0.27^{\circ}$$

$$29.0^{\circ} \pm$$

\* Minimum time of sporulation of *Saccharomyces pastorianus* (Hansen, 1883).

† Minimum time of sporulation of *Saccharomyces cerevisiae* (Hansen, 1883).

A critical temperature at 15° has not been obtained previously for respiration of yeast, so far as I have been able to find from published experiments. Slator's data (1906, 1908) give critical temperatures at 20.2°, 22.7°, and 35-40°(?). These values cannot be taken as especially significant, because the original data, as has been noted already, are not really accurate enough for use in a temperature analysis. The data of Kubowitz (1929) for O<sub>2</sub> consumption of baker's

yeast over the range  $-3.5$  to  $+37.5^{\circ}\text{C}$ . when plotted according to the Arrhenius equation show evidence of critical temperatures at  $11.4^{\circ}$  and above  $30^{\circ}\text{C}$ . Unfortunately the determinations were made at so few temperatures (six in all) that any evaluation of  $\mu$  (or of critical temperatures) must be based on two points in the upper temperature range and three in the lower range and consequently is of doubtful significance.

$19.5\text{--}20^{\circ}\text{C}$ . was found by Tang (1930-31) to be a critical region for the  $\text{O}_2$  consumption of germinating seeds of *Lupinus albus* and of *Zea mays*. Navez (1928-29) found  $21.0^{\circ}$  to be a critical temperature for  $\text{CO}_2$  production by seedlings of *Vicia faba*. Recently Crozier and Navez (1930-31) assigned  $20.0^{\circ}$  as the critical temperature for the  $\text{CO}_2$  production by seedlings of *Phaseolus aureus* Roxb. The  $\text{CO}_2$  production by seedlings of *Pisum* yields a critical temperature at  $21.0^{\circ}\text{C}$ . (data of Kuyper, analyzed by Crozier, 1924-25; and Navez, 1928-29). Tang (1931-32) found  $20^{\circ}\text{C}$ . to be a critical temperature for the  $\text{CO}_2$  production by *Lupinus albus*, but he found no evidence of a critical temperature for the  $\text{CO}_2$  production by *Zea mays* in the range of temperatures studied.

$30^{\circ}$  is a definite critical zone for yeast. My experiments on  $\text{O}_2$  uptake show that this is a critical temperature. Richards (1928) showed that the rate of growth of yeast decreases very rapidly above this temperature and that definite morphological changes occur in the budded cells growing at  $30^{\circ}$ . The usually roundish cells found below  $30^{\circ}$  now grow as "irregular elongate cells" above  $30^{\circ}$ . Just how the mechanism producing such cells may be related to the value  $\mu = 8,000$  found for  $\text{O}_2$  consumption above  $30^{\circ}$  has not been ascertained as yet. Estimations by Hansen (1883, data taken from a paper by Herzog (1902-03)) of the time of production of the first ascospore in suspensions of yeast give an apparent critical temperature in the region of  $30^{\circ} \pm$  for *Saccharomyces cerevisiae* and in the zone of  $27^{\circ} \pm$  for *Saccharomyces pastorianus*. However, these values are of doubtful significance since they are obtained from an Arrhenius plot in which the data gave a curvilinear relationship.

In view of these facts one should be cautious when choosing a temperature for comparing effects of substances on rates of respiration. The region  $30\text{--}37^{\circ}$  has been used most frequently for such determinations. Temperatures below  $30^{\circ}$  would be nearer the range of "normal" physiological activity for yeast and similar organisms.

#### d

The interpretation of the temperature characteristics obtained for  $\text{O}_2$  uptake of yeast in the presence of dextrose is briefly as follows: it



is thought that a value of  $\mu$  refers to, and characterizes, the slowest or the governing process, under the conditions, in the chain of respiratory processes (*cf.* Crozier, 1924, 1924-25). At a critical temperature a new reaction becomes the "master reaction" (*cf.* Blackman, 1905; Crozier, 1924-25), and so limits the over-all rate of the processes concerned with the consumption of  $O_2$ .<sup>4</sup>

Difficulties confronting this interpretation are realized, and have already been stated (p. 815). However, by studying the kinetics of metabolic processes as a function of independent variables such as temperature, oxygen pressure, etc., and making use of methods already indicated, one may expect to establish the identity of these "master reactions" and their controlling catalysts, and then to formulate a more complete picture of the organization of the processes resulting in the uptake of oxygen.

I wish to thank Professor Barcroft, and other members of the Physiological Laboratory, for their kindness and helpfulness to me during my sojourn at Cambridge University as a National Research Fellow.

#### SUMMARY

Suspensions of the yeast *Saccharomyces cerevisiae* gave reproducible rates of  $O_2$  uptake over a period of 6 months. The relation of rate of consumption of  $O_2$  to temperature was tested over a wide range of temperatures, and the constant in the formulation of the relationship is found to be reproducible. The values of this constant ( $\mu$ ) have been obtained for five separate series of experiments by three methods of estimation. The variability of  $\mu$  has the following magnitudes: the average deviation of a single determination expressed as per cent of the mean is  $\pm 2$  per cent in the range  $30-15^\circ$ , and  $\pm 0.8$  per cent in the range  $15-3^\circ C$ . This constancy of metabolic activity measured as a function of temperature can then be utilized for more precise investigations of processes controlling the velocity of oxidations of substrates, and of respiratory systems controlled by intracellular respiratory pigments.

<sup>4</sup> Experiments by the author (to be published shortly) on the relation of temperature to the time for reduction of cytochrome give additional evidence supporting this interpretation.

The data plotted according to the Arrhenius equation give average values of the constant  $\mu$  as follows: for the range  $35-30^{\circ}$ ,  $\mu = 8,290$ ;  $30-15^{\circ}$ ,  $\mu = 12,440 \pm 290$ ;  $15-3^{\circ}$ ,  $\mu = 19,530 \pm 154$ . The critical temperatures are at  $29.0^{\circ}$  and  $15.7^{\circ}\text{C}$ .

A close similarity exists between these temperature characteristics ( $\mu$ ) and values in the series usually obtained for respiratory activities in other organisms. This fact supports the view that a common system of processes controls the velocities of physiological activities in yeast and in other organisms.

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# DEVELOPMENT OF THE EGG OF THE MACKEREL AT DIFFERENT CONSTANT TEMPERATURES

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That an increase in the temperature of the medium, within limits, accelerates the embryonic development of fishes has been adequately demonstrated by several investigators. This was studied by Dannevig (1895), by Reibisch (1902), by Apstein (1909), and by Johansen and Krogh (1914) in several species of *Pleuronectes* and in *Gadus morrhua*. The development of two Japanese fishes, *Hypomesus olidus* and *Plecoglossus altivelis*, has been observed from this standpoint by Higurashi and Tauti (1926), and Kawajiri (1927) records similar data for the Japanese trout (*Oncorhynchus mason*).

The development of the mackerel (*Scomber scombrus* L.) has not previously been examined from this point of view. A detailed study of the relation of temperature to the rate of development of the egg of this fish from the time of its fertilization until hatching was undertaken during the mackerel spawning seasons of 1931 and 1932 at the Woods Hole, Massachusetts, Station of the United States Bureau of Fisheries.

It was especially desirable to record as accurately as possible the time necessary at different constant temperatures for the attainment of various embryonic stages of development, as well as the time required for hatching. Secondly, it was interesting to determine the upper and lower temperature limits for typical development. Some information regarding mortality and survival at different temperatures was also obtained.

## Methods

Ordinary fish hatching boxes were employed as constant temperature water baths. Reasonable insulation for the thermostat is afforded by the walls of the

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\* Published with the approval of the Commissioner of Fisheries.

box, which are composed of wood an inch in thickness. Regulation of temperature within each tank was obtained by use of a mercury thermoregulator equipped with a nichrome needle. This actuated a relay-controlled heating lamp or series of lamps. 12 inch, 40 watt show-case lamps were used for this purpose.

An ice funnel of the type described by Stier (1931) was used as a cooling unit in each thermostat. The hopper of each of these was made of battleship linoleum and made 3 feet in length so that at hatchery temperatures during the month of June it was necessary to replace ice only every 12 or 16 hours. The funnel was supported at the desired level in the bath by means of a horizontal rod clamped at each end to a large stand. This arrangement was found somewhat more satisfactory than a pulley system and counterweight since the funnel was held much more firmly in place. Washing of the chopped ice before use prevented the accumulation of debris at the sieve in the funnel. Water from melting ice was rapidly drained off through a rubber tube leading to an opening in the floor of the box.

A single motor and propeller mounted vertically served as a stirrer and produced a satisfactory water circulation in each thermostat.

Ripe adult mackerel were collected at fish traps and transported alive to the laboratory and stripped. Eggs were fertilized artificially and placed in 250 cc. beakers nearly filled with sea water of the temperature at which fertilization had been effected. During the half-hour subsequent to fertilization the beakers containing the eggs were lowered into the baths and supported with their mouths at the surface on ring-stands.  $\frac{1}{2}$  to  $\frac{3}{4}$  of an hour was required for the beaker water to assume the desired temperature after the beakers had been placed into the thermostats. Almost without exception 15 cc. of eggs (approximately 13,000) were placed in each beaker, but in a few instances the scarcity of eggs prevented this. Usually two beakers containing eggs were placed in each thermostat at each temperature.

Air, of the temperature of the bath, was bubbled through the water of each egg beaker at a slow and nearly constant rate. Air pressure was kept constant by means of a simple water manometer at the end of the main air line, and a screw clamp on each secondary air line above each beaker was used to regulate the air outflow. Care was taken to avoid churning of the water in the beakers, so that the eggs would not suffer injury. The air passed through a bottle of sea water before reaching the egg beakers.

The living mackerel egg is pelagic, while dead eggs sink. This fact greatly facilitated the daily removal of dead eggs from the hatching beakers by means of a large pipette. Dead eggs were roughly counted at the time of their removal and the data secured in this way were used in obtaining figures concerning mortality and survival. Water in the beakers was replaced each day with fresh sea water which had previously been brought to thermostat temperature.

Eggs were developed in baths of constant temperature of 9°, 10°, 11°, 12°, 13°, 14°, 15°, 16°, 17°, 18°, 19°, 20°, 22°, and 24°C. Temperatures of 10°, 12°, 14°, 15°, 16°, 21°, 22°, and 24° were successfully run during June, 1931. Experiments at 11°, 18°, and 20° were spoiled. During June, 1932, these experiments were all

repeated with the exception of the 24° temperature, and lots of eggs were run at 9°, 13°, 17°, and 19° in addition. Beckman thermometer readings taken twice each day showed that the water temperature in a single thermostat beaker varied within limits of 6/100°C. during the several days required for the completion of an experiment.

The stage of development of each of ten eggs in each of the thermostats was observed at intervals of about 2 hours during the day—less frequently at night—during the period of incubation. In the early cleavage stages observations were made every half hour. Eggs removed from the thermostats for observation were not replaced.

### *The Normal Development of the Mackerel Egg*

The development of the mature, fertilized mackerel egg is very rapid and hatching may take place in as short a time as 48 hours at high temperatures. The development of the embryo mackerel is similar to that of the sea bass (*Serranus atrarius*) which has been described in considerable detail by Wilson (1891). For this reason only a brief description will be given here.

The fertilized egg divides first into 2, then 4, 8, 16, 32, 64, and 128 cells. These stages are very easily identified and were used as landmarks of early development in these experiments. They are, however, passed through so quickly that they are of little use in this respect. At low temperatures, when development proceeds relatively slowly, a six cell stage may occur and a twelve cell stage may not infrequently be observed.

Following the 128 cell stage, the cell walls of the peripheral cells of the blastoderm gradually disappear and the nuclei of these cells escape into the periblast, undergo further division, and finally there is formed a periblast ridge as a result. While the periblast nuclei are multiplying and spreading over the yolk, the marginal cells of the blastoderm proper divide to form a "*randwulst*" which appears first at one point and gradually extends until the entire blastodisc is surrounded by a germ ring. The polarity of the blastoderm is expressed by a thickening of the germ ring at its dorsal margin. This thickening subsequently develops into the axial portion of the embryo proper. The first appearance of disappearing peripheral cell walls, the first appearance of periblast nuclei, the completion of the periblast ridge, the appearance of the "*randwulst*," the completion of the germ ring, and the appearance of the head fold have been considered fairly definite landmark stages in the development of the mackerel.

Continued ingrowth of the anterior end of the blastoderm is accompanied by involution and epiboly at the posterior end and at this time the embryo appears from the surface to be undergoing confluence. The embryo by this period has a definitely elongate shape, extending for about 90° around the yolk, and epiboly or overgrowth of the yolk is about half completed. Epiboly continues until the cells

of the germ ring push in from all sides at the posterior end and leave only a small opening, the blastopore, which then itself becomes closed and epiboly may be said to be complete. Almost simultaneously, the anterior end of the embryo proper has undergone an extent of differentiation resulting in the formation of the primordia of the optic vesicles. The stages during epiboly used as landmarks in this study have been called "half epiboly," "three-quarter epiboly," and "completed epiboly," or "blastopore closure."

Somites or primitive muscle segments now become apparent in the body of the embryo. These multiply rapidly and their number at any time may be used as an index of the stage of development of the embryo as a whole. At 6 somites, melanophores make their first appearance; the heart becomes distinct at 10 somites and begins to show movement at 24 somites. Chromatophores appear on the head at 18 somites. The following terms have been used to indicate the stages of development just described: "6 somites," "12 somites," "18 somites," and "24 somites."

After the heart begins to beat the somites become too numerous to be counted with ease, and then I have considered the length of the embryo as compared with the circumference of the egg the most satisfactory criterion of the progress of development. At 24 somites the embryo in length covers somewhat less than two-thirds of the circumference of the egg, but continues to increase in length until it forms a complete circle, and some time before hatching, the tail actually overlaps the head for a considerable distance. Hatching merely involves the rupturing of the chorion which permits the escape of the larva. The following landmark stages are frequently referred to in this paper: "two-thirds circle," "three-fourths circle," "four-fifths circle," "five-sixths circle," "full or completed circle," and "hatching." This study of the development of the mackerel has considered only the changes of the embryo which take place between the time of fertilization and hatching.

### *Temperature Limits for Normal Development*

The spawning season of the mackerel at Woods Hole, during 1931 and 1932 embraced the period from about May 15 to June 20. The sea water temperature at Woods Hole during this period increased from about 11.5° to 18°C.

Early June experiments demonstrated that mackerel eggs fail to cleave normally at temperatures as low as 10°, although they develop in a typical manner at 11°C. Embryos which were held at a thermostat temperature of 8° displayed no development whatever, although eggs were kept under observation at this temperature for approximately 48 hours. The nuclei of eggs held at 8.5° underwent division as many as four times, so that 16 nuclei resulted in each egg, but there was no cytoplasmic division. No further development was noted.

At 9°C. the eggs cleaved irregularly up to the 128 cell stage; nuclei multiplied in the periblast and eventually an irregular mass of cells resembling a yolk cap with a completed periblast ridge formed, composed of irregular and relatively few cells. No progress was observed at this temperature during the subsequent 48 hours and it was concluded that development had ceased. At a constant temperature of 10°C., cleavage was irregular and obscured, making it impossible to identify the stages of development until the appearance of the optic vesicles. Development, however, continued at the expected rate up to 18 somites. Then it began to slow down and although some of the embryos attained the approximation of a five-sixths circle stage, the appearance of this stage was very much delayed. None of the embryos developed beyond this point and all finally died. The length of the period of development at this temperature up to hatching was computed from the time necessary for the attainment of 18 somites at this temperature as compared with the time necessary for the approximation of the same stages at 11°C. Typical cleavage and development were secured at a thermostat temperature of 11°C. although the mortality was very great.

Eggs failed to undergo typical development at temperatures above 21°C. At 22°, for example, although a few of the eggs appeared to develop in a normal manner up to the time of the appearance of the head indentation, the majority cleaved in an irregular fashion and all were dead 22 hours after fertilization. Similar results were observed at 24°C.

It was concluded that during the month of June at Woods Hole, typical development of mackerel eggs can be secured only between temperatures of 11° and 21°C. Temperatures above and below these limits have resulted in abnormal development under experimental conditions.

#### *Mortality and Survival of Mackerel Eggs at Different Temperatures*

Mortality of mackerel eggs under laboratory conditions is great. Dead eggs were removed from the beakers used for egg hatching in the thermostat every 24 hours and measured in cubic centimeters or counted directly. At all temperatures the percentage reaching hatching was low.



At temperatures of 9°, 10°, 21°, 22°, and 24°C. the mortality during the incubation period amounted to 100 per cent. A certain percentage of survival was secured at all temperatures included within these limits. The percentage of survival was greatest at 16° during both 1931 and 1932, where the mortality averaged 43 per cent. Between this optimum temperature and the extreme high and low temperatures there was a progressive increase in mortality. This is recorded in Fig. 1, in which the total percentage of eggs dying at each temperature is given; no data were secured at 17° or 19°.

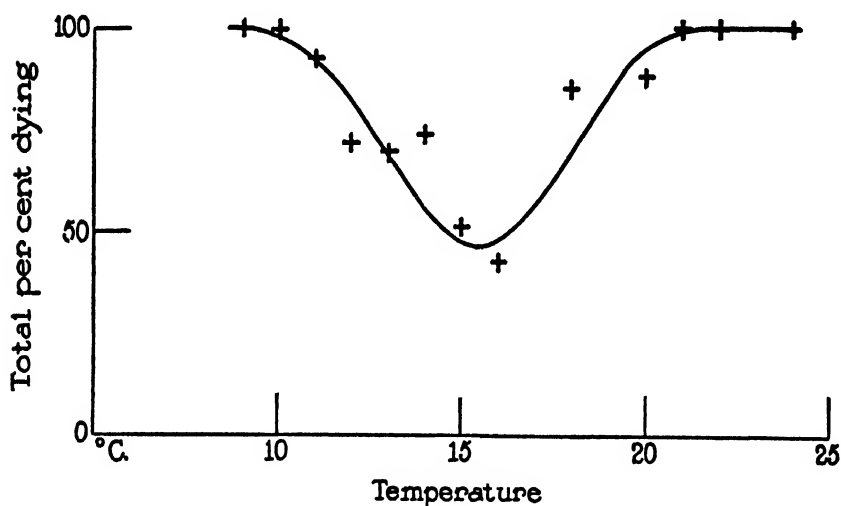


FIG. 1. Mortality of mackerel eggs at different constant temperatures. The total percentage of eggs dying is plotted along the ordinates; the temperatures along the abscissae.

Eggs placed in 9° thermostats all died within 96 hours. At this temperature, development, if completed, would be expected to occupy a period of approximately 9 days. These embryos were, therefore, killed before development was half completed and the greatest percentage of mortality occurred during the third 24 hour period. It was found impossible to determine from an examination of the eggs at what stage death had occurred, and even the stage of development of the living eggs could not be told, due to atypical cleavage and obscured development.

100 per cent mortality was experienced at 10°C. although a few eggs

TABLE I

*Mortality of Mackerel Eggs at Different Temperatures*

In this table, the percentage of the total number of eggs originally placed in thermostats that died during each 24 hour period of incubation during 1931 and 1932, is given.

Temperature	Season	Dying 1st day	Dying 2nd day	Dying 3rd day	Dying 4th day	Dying 5th day	Dying 6th day	Dying 7th day	Dying 8th day	Total dying
°C.		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
9	1932	30	8	49	13	—	—	—	—	100
10	1931	17	17	66	—	—	—	—	—	100
	1932	10	40	50	—	—	—	—	—	100
		40	14	26	6	3	3	6	3	100
11	1932	25	10	5	5	5	15	25	—	90
		10	5	10	5	5	40	20	—	95
12	1931	13	33	17	6.5	6.5	10	—	—	86
	1932	17	13	13	3	0	13	—	—	59
13	1932	10	10	5	5	0	30	—	—	60
		15	5	5	10	20	25	—	—	80
14	1931	13	40	7	10	13	—	—	—	83
	1932	17	17	4	11	17	—	—	—	66
15	1932	20	18	3	10	—	—	—	—	51
16	1931	20	13	7	13	—	—	—	—	53
	1932	20	10	7	—	—	—	—	—	37
		27	13	0	—	—	—	—	—	40
18	1932	10	10	66	—	—	—	—	—	86
20	1931	46	23	27	—	—	—	—	—	96
	1932	47	30	—	—	—	—	—	—	77
		53	37	—	—	—	—	—	—	90
21	1932	93	7	—	—	—	—	—	—	100
		93	7	—	—	—	—	—	—	100
22 and 24		100	—	—	—	—	—	—	—	100

were well into the circle stages before they died. The majority of the eggs died during the period of germ ring formation which fell

during the third 24 hour period of incubation. The somite multiplication periods were also stages of high mortality at this temperature.

At 11°C. the period of germ ring formation and the somite multiplication stages again were periods of high mortality and this was also the case at 12°, 13°, 14°, and 15°C. At 20°, 21°, 22°, and 24°, the stages of germ ring formation and epiboly come within the first 24 hour period of incubation and the greatest percentage of mortality falls here as well. At 22° and 24°, 100 per cent mortality appears during the first 24 hour period. Apparently the stages of germ ring formation and epiboly and the somite multiplication stages are critical periods in the development of the embryo of the mackerel. Table I summarizes the data on the mortality of eggs; the percentage of the total number of eggs originally placed in the thermostats that died during each 24 hour period of incubation is given for each temperature. The total percentage dying is recorded in the final column.

#### *Determination of the End-Point of Incubation*

Mackerel eggs which have all been fertilized at the same time and allowed to develop at the same temperature in the same thermostat do not hatch simultaneously. This "hatching period" may extend over many hours, particularly at low temperatures. At higher temperatures it is much shorter; there is a progressive increase in the length of the hatching time with a decrease in temperature. At temperatures ranging between 20° and 17° the hatching time amounts to between 3 and 6 hours; between 16° and 14° it occupies from 6 to 10 hours; at 13° and 12° approximately 12 hours are required; while at 11°, 16 or more hours are necessary.

It was found advisable to adopt some consistent method for the determination of the end-point for incubation, applicable to all temperatures. The method adopted involved the plotting of a frequency curve for hatching and the use of the time corresponding to the crest of the curve as the end-point. To obtain an unbroken frequency curve it is necessary to note the time when the first larva hatches and to count the number hatching per unit time until hatching is completed. Half-hour periods were used as units of time for the higher temperatures, while at temperatures between 11° and 14° units an hour in length were found more satisfactory. Complete curves were obtained for only a few temperatures, although in all cases the crest of the curve was accurately determined. Often it was not discovered that hatching had commenced until sometimes as much as 2 hours afterward and the initial portion of the curve suffered as a consequence. In some

instances, too, it was found impossible for lack of time to continue observations up to the time of hatching of the last embryo and the final portion of the curve for that particular temperature was not completed. Another difficulty of some importance expressed itself occasionally, for not infrequently at the height of hatching eggs hatched more rapidly than they could be counted. In such cases, as accurate an estimation of the number was made as the time allowed.

For the most part, satisfactory results were secured in plotting frequency curves for hatching at various temperatures and no difficulty was encountered in finding

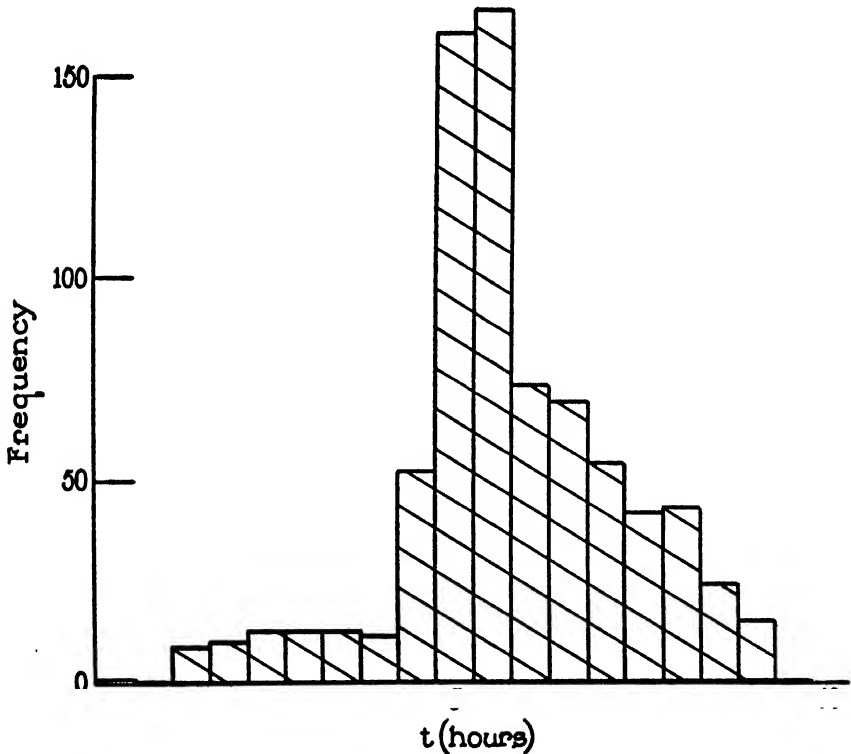


FIG. 2. Hatching data at 16°C. The number hatching each half hour following the initiation of hatching is plotted.

the mode of any particular curve when, as was usually the case, a large number of eggs was carried through to hatching. A histogram of the data secured at 16° is given in Fig. 2. This is fairly typical of the results obtained at all temperatures.

#### *Rate of Development to Hatching of Mackerel Eggs at Different Constant Temperatures*

Between 11° and 21°C. the time required for the development of mackerel eggs from the time of fertilization to hatching decreases from

177 hours to 49½ hours. In other words, such an increase in temperature more than trebles the speed of development. The  $Q_{10}$  figure for the process is, therefore, about 3.6, indicating, in accordance with the principle of van't Hoff (1884), that the reaction is of a chemical nature. The  $Q_{10}$  ratio is, however, not constant throughout the temperature range from 10–21° as Table II indicates.

It was originally planned to carry mackerel eggs only once at each constant temperature within the limits of tolerance for this species, but time was found to repeat during 1932 some of the experiments of the previous year as a check on the early results obtained. Temperatures of 12°, 14°, 15°, 16°, and 21° were repeated in 1932. Practically

TABLE II  
*Q<sub>10</sub> Values for Mackerel Egg Hatching*

Temperature limits	$Q_{10}$ value
°C.	
10–20	3.66
11–21	3.57
10–15	4.16
11–16	4.06
12–17	3.90
13–18	3.76
14–19	3.46
15–20	3.52
16–21	3.52

perfect agreement with the 1931 results was secured at the 16° and 21° temperatures, but discrepancies were noted in the others. The modes of hatching times in the two experiments carried on at 12° fell 4 hours apart. This may be due to the fact that the incubation period and the period of hatching are so long at this temperature.

No great agreement could be obtained with regard to the length of the incubation period at temperatures of 14° and 15°, although these experiments were repeated twice in the first case and carried on four times in the second instance. At 14° the hatching modes in three experiments fell at 105, 109, and 117 hours after fertilization; at 15°, the modes for hatching fell at 93, 99, 101, and 101½ hours respectively after the time of fertilization, in four experiments at this temperature.

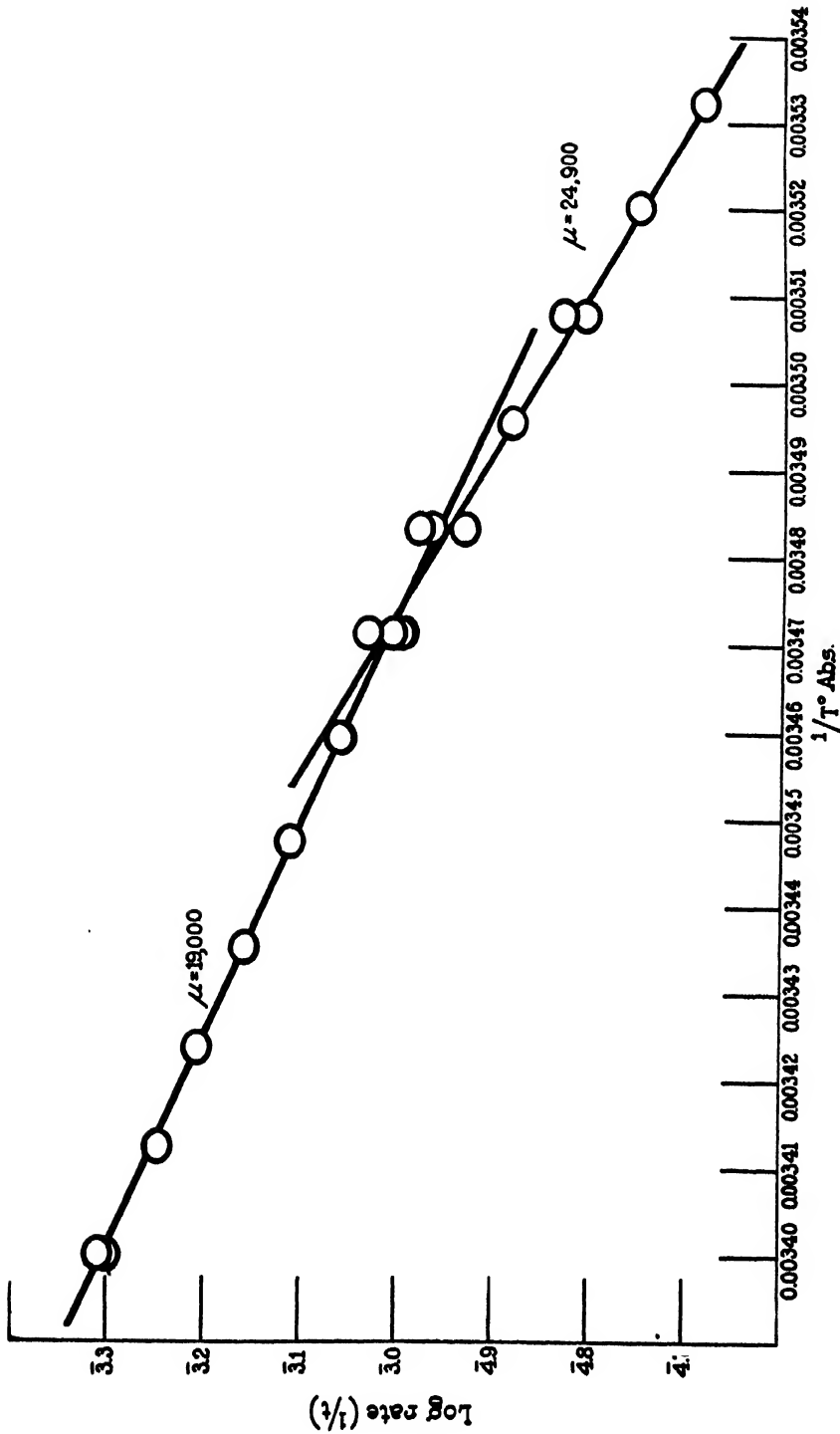


FIG. 3. The rate of development to hatching of mackerel eggs as a function of temperature

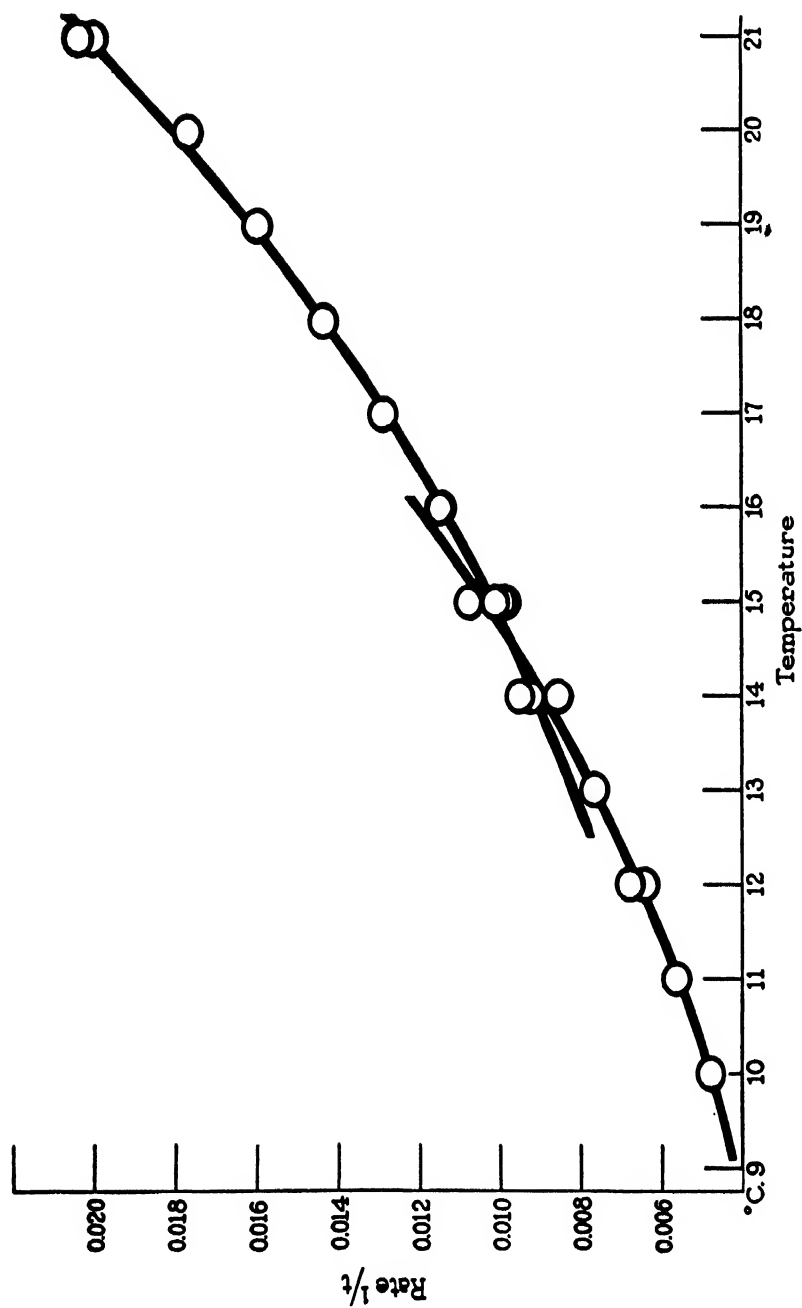


FIG. 4. The rate of development to hatching of mackerel eggs as a function of temperature; the curves drawn are the straight lines which appear in Fig. 3.

The variation in the length of the incubation period at these two temperatures may be due to the fact that these temperatures are so close to a critical temperature for many biological processes which is said to occur in the vicinity of 15°C. (Crozier, 1926), and in the present case for rate of development (Fig. 3). Figs. 3, 4, and 5 demonstrate the relation of temperature to the rate of development to hatching of mackerel eggs.

The log of the rate of development to hatching ( $\log 1/t$ ) is plotted against the reciprocal of the absolute temperature ( $1/T^\circ \text{ abs.}$ ) in Fig. 3. Two straight lines, intersecting in the vicinity of 15°, are apparent. The calculated  $\mu$  for the rate of hatching above 15° is found from the curve to be approximately 19,000 calories. Below 15° a value  $\mu = 24,900$  is obtained. These values of  $\mu$  coincide relatively well with values previously obtained for the development of organisms including fishes (Crozier, 1926). The rate of development to hatching ( $1/t$ ) is plotted against the temperature in degrees Centigrade in Fig. 4. The graph consists of two curves which intersect near 15°. In Figs. 3 and 4, 2 points are plotted for 12°, 3 for 14°, 4 for 15°, 2 for 16°, and 2 for 21°. When more than one point is at hand for a certain temperature, the curve has been drawn as nearly as possible through the average of the several points plotted from the data.

#### *Rate of Attainment of Various Embryonic Stages at Different Constant Temperatures*

Considerable attention has been devoted to the length of time necessary for the attainment of various embryonic stages in development. Twenty-eight stages in development, including hatching, have been considered. These have been listed above. Figs. 5 and 6 summarize these data.

The time necessary for the attainment of various embryonic stages at different temperatures is displayed in Fig. 5. The points indicated in this graph are averages in many cases of two or more readings. In many cases, however, the point plotted represents but one observation. Observations are lacking in the case of some stages at certain temperatures and points are omitted on the graph in consequence. A missing reading indicates that observations fell before or following the approximation by the eggs of the stage in question.



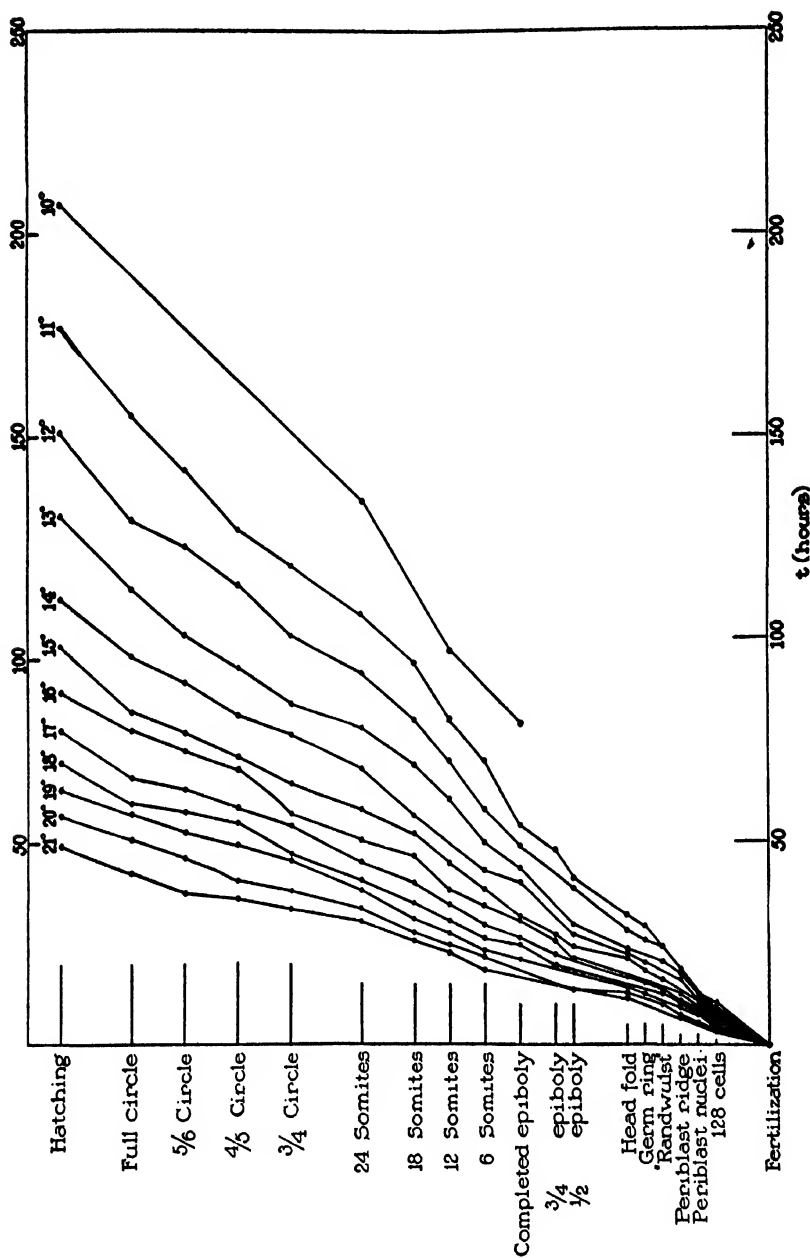


FIG. 5. The velocity of development of mackerel eggs at different constant temperatures. The average time necessary for the attainment of various embryonic stages is plotted for each temperature.

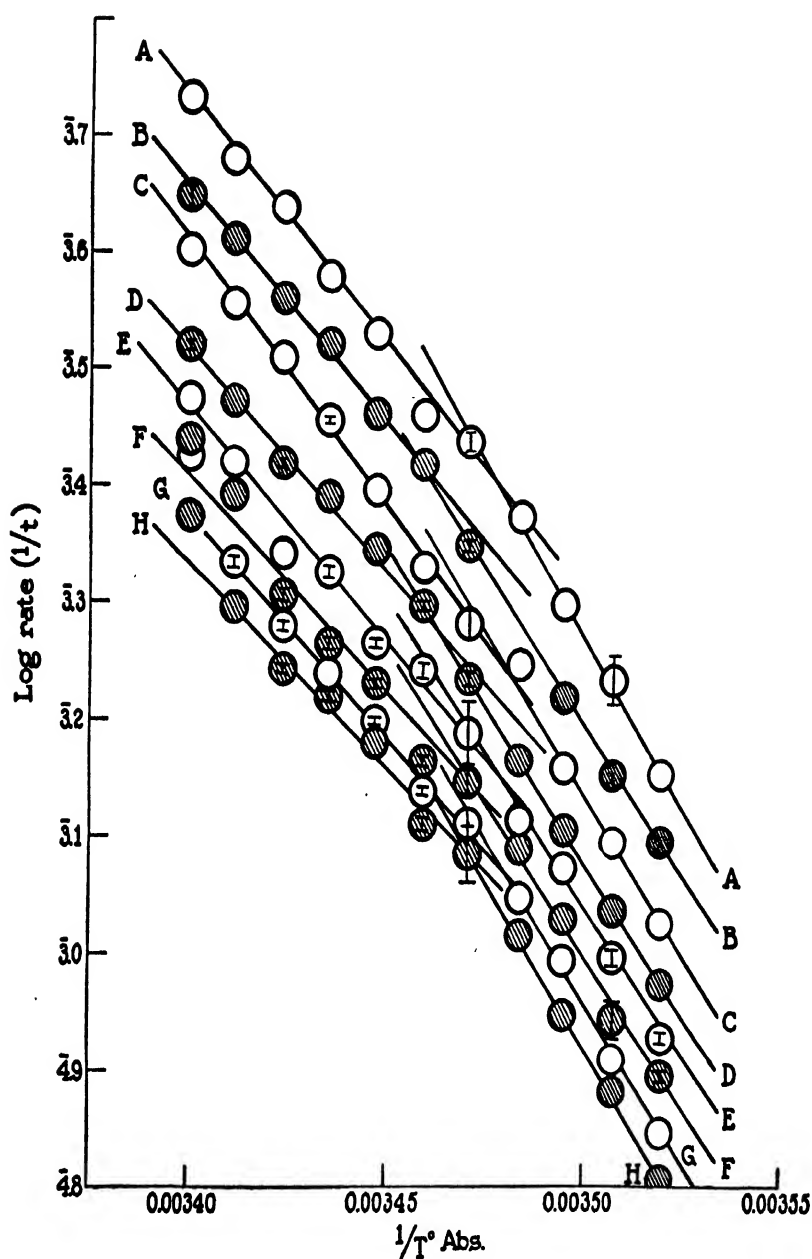


FIG. 6. Data on the development of the mackerel. Eight different end-points have been selected for each temperature:

- |                           |                        |                        |
|---------------------------|------------------------|------------------------|
| A. Full circles.          | B. Five-sixth circles. | C. Four-fifth circles. |
| D. Three-quarter circles. | E. 24 somites.         | F. 18 somites.         |
| G. 12 somites.            | H. 6 somites.          |                        |

The stage of development of each of ten embryos was noted at the time of each observation. Usually, especially after development had proceeded for several hours, more than one stage was represented in a single lot of eggs at one time. The points plotted in Fig. 5 represent the mean times. The stages in development are listed here in order along the ordinate and the time required to reach each of these stages is plotted on the abscissae for each temperature. The points representing any given temperature are connected. The fact that this graph is based upon actual observations only, accounts for the failure of the lines connecting points to follow an entirely even course. The time between successive observations sometimes amounted to several hours, particularly at night; consequently, the time recorded as necessary for the attainment of a given stage may actually be too great by an amount almost equal to the length of time between successive observations.

Thermal increments have been calculated for the speed of arrival at each of eight of the later embryonic stages; *i.e.*, 6 somites, 12 somites, 18 somites, 24 somites, three-quarters circle, four-fifths circle, five-sixths circle, and full circle. These are displayed in Fig. 6. Probable errors are indicated for as many points as the data allowed. There is no great disagreement in the values of  $\mu$  obtained for these stages and those manifest from the hatching data within the temperature limits used. The curves for the different stages plotted are essentially parallel. The few discrepancies may, perhaps, be accounted for by the fact that the end-points for these stages were not as carefully determined as were those for hatching. The critical temperatures for each stage fall in the vicinity of  $15^{\circ}$ . It will be noted that the probable errors are greatest at this temperature. The close agreement of  $\mu$  values for developmental stages and those of hatching indicates that the temperature characteristics for "average rates of development" are not influenced by the end-points chosen. The rate of differentiation up to hatching seems to be governed by one process throughout.

#### SUMMARY

1. Mackerel egg development was followed to hatching at constant temperatures of  $10^{\circ}$ ,  $11^{\circ}$ ,  $12^{\circ}$ ,  $13^{\circ}$ ,  $14^{\circ}$ ,  $15^{\circ}$ ,  $16^{\circ}$ ,  $17^{\circ}$ ,  $18^{\circ}$ ,  $19^{\circ}$ ,  $20^{\circ}$ ,  $21^{\circ}$ ,

22°, and 24°C. Experiment showed that typical development could be realized only between 11° and 21°.

2. The length of the developmental period increases from 49.5 hours to 207 hours when the temperature is lowered from 21° to 10°C.

3. The calculated  $\mu$  for the development of the mackerel egg is about 19,000 at temperatures above 15° and approximately 24,900 for temperatures below 15°C. 15° is, apparently, a critical temperature for this process.

4. The calculated values of  $\mu$  for eight stages of development preceding hatching, *i.e.* 6 somites, 12 somites, 18 somites, 24 somites, three-quarters circles, four-fifths circles, five-sixths circles, and full circles, are essentially the same as the  $\mu$ 's for hatching, indicating that the rate of differentiation up to hatching is governed by one process throughout. Critical temperatures for these stages approximate 15°.

5. The total mortality during the incubation period was least at 16°C. where it amounted to 43 per cent. At temperatures above and below this there was a steady increase in the percentage of mortality which reached 100 per cent at 10° and 21°.

The author wishes to express his thanks to Mr. O. E. Sette, at whose suggestion this study was undertaken, to Professor Leigh Hoadley, Dr. P. S. Galtsoff, Dr. T. J. B. Stier, and others for suggestions made during the course of these investigations, and to Professor W. J. Crozier for assistance in preparing the manuscript for publication. Thanks are due also to many other members of the staff of the United States Bureau of Fisheries for assistance of various kinds.

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# THE VIOLOGEN INDICATORS

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## INTRODUCTION

The quaternary bases derived from  $\gamma, \gamma'$ -dipyridyl have proven to be useful as oxidation-reduction indicators of properties very desirable for biological purposes, especially because their potential range is very negative, under certain conditions more negative than that of any member of the series of indicators worked out by W. M. Clark and his associates (1) and supplemented by various other authors. The new indicators will be designated as *viologens* according to a proposition made in a preliminary communication (2). They differ from other indicators in several respects; in the first place, the color is exhibited by the reduced form, whereas usually the oxidized form is the colored one.<sup>1</sup> Secondly, the oxidation-reduction potential of these substances is independent of pH. In a neutral solution the normal potential is very close to the potential of a hydrogen electrode at the same pH, therefore in an alkaline solution the potential is more positive, and in an acid solution it is more negative than the hydrogen potential at the same pH. Therefore, in acid solution their normal potential is a hydrogen overvoltage, in other words, rH is negative.

The preparation of one representative of these indicators has been briefly described previously (2, 4). Several improvements and simplifications in the preparation of this compound and the preparation of various homologous compounds, and their properties as regards oxidation-reduction potential, as well as their optical properties, will be described in this paper.

<sup>1</sup> Also the anthraquinone sulfonates, studied by Conant, Kahn, Fieser, and Kurtz (3), have a more intense color in the reduced state than in the oxidized. Their color intensity, however, is rather small even in the reduced state.

### *Preparations*

It has been known for a long time that  $\gamma, \gamma'$ -dipyridyl can attach two molecules of an alkyl-halide thus forming a bi-quaternary base. The method of preparation consisted in preparing first a quaternary base of pyridine and condensing two molecules of it to a dipyridyl compound by treatment with sodium amalgam and successive oxidation. As early as 1881, A. W. Hofmann (5) prepared dibenzyl-dipyridylium iodide in this way. He considered his preparation as an  $\alpha, \alpha'$ -dipyridyl derivative. Much later Emmert (6) proved it to be a  $\gamma, \gamma'$ -dipyridyl compound. The yields of this method are poor. Since Dimroth and Heene (7) discovered a convenient method of preparing  $\gamma, \gamma'$ -dipyridyl it seemed more convenient to use this compound as the parent substance for the preparation of the bi-quaternary bases. The following procedure of preparing  $\gamma, \gamma'$ -dipyridyl is essentially that followed by Dimroth and Heene, and Dimroth and Frister (7, 8).

(a) *Preparation of  $\gamma, \gamma'$ -Dipyridyl.*—50 cc. pyridine, dried over barium oxide, 250 cc. acetic anhydride, and 50 gm. zinc dust are either shaken by machine for 6 hours in a stoppered bottle, or stirred mechanically for 2 hours in a beaker. The yellow precipitate developed is dissolved as well as possible by heating the mixture in a water bath at 90°C. for 1–2 hours. The liquid is filtered off and allowed to crystallize in the ice box. The yellow crystalline precipitate is collected on a Büchner filter, without washing, and exposed to the air in a flat dish at 50°C. It gradually changes in a not easily reproducible way. Sometimes it becomes light brown and within several days the colorless needles of  $\gamma, \gamma'$ -dipyridyl crystallize out within the brown substance. Sometimes the substance becomes brown altogether. Usually the whole mass becomes liquescent and later dries out again. According to the course of these changes, the final yield of  $\gamma, \gamma'$ -dipyridyl may vary widely. In any case, after the substance has been exposed in this way to the air for several days it is boiled with 200 cc. water and enough sodium hydroxide added to make the solution strongly alkaline. It is filtered hot and allowed to crystallize in the ice box. Then  $\gamma, \gamma'$ -dipyridyl crystallizes in long, white needles and may be recrystallized by dissolving in hot, slightly acidified water and precipitated by sodium hydroxide. The crystals contain two molecules of water, which they lose on drying at 50°. The melting point of the anhydrous substance is 111.0–112.0°C.

A test for  $\gamma, \gamma'$ -dipyridyl is as follows: a small amount is dissolved in 50 per cent acetic acid and some solid chromous chloride is added.

An intense violet color is developed, which fades on shaking in the air. Instead of chromous chloride, zinc dust may be applied. In this case the violet color is only transient and the reduction goes further, to a colorless state. This second step of reduction is irreversible.

(b) *N-N'-Dimethyl- $\gamma,\gamma'$ -Dipyridylum-Bichloride (or Methyl Viologen)*.—10 cc. dimethyl sulfate and 1 gm.  $\gamma,\gamma'$ -dipyridyl are mixed and heated over a free flame just to the beginning of full boiling. After spontaneous cooling the heating is repeated once in the same way. The mixture is poured into a separatory funnel and 50 cc. water added. The excess of methyl sulfate is removed by repeated extraction with ether. The aqueous solution is poured into a beaker and an excess of a concentrated picric acid solution is added. The crystals of the picrate are collected on a Büchner filter, washed with a little acetone, sucked nearly dry, and suspended in 100 cc. acetone. A small amount of concentrated hydrochloric acid is added (1.0 cc.). Gradually the yellow picrate is converted into the colorless chloride which is insoluble in acetone. The crystals are collected and may be recrystallized by dissolving in a small amount of methanol and precipitating with a large excess of acetone. The substance forms perfectly colorless needles, which, on drying at 50°C., lose the water of crystallization. The yield is almost the theoretical one. The substance, dissolved in water, on adding sodium hydrosulfite and ammonia, turns deep violet; in higher dilution it is a pure blue, in higher concentration a little more violet. ( $\gamma,\gamma'$ -Dipyridyl itself does not give this test but develops color only when reduced in an acid medium according to the above directions.)

(c) *N-N'-Diethyl- $\gamma,\gamma'$ -Dipyridylum-Bichloride (or Ethyl Viologen)*.—This compound is prepared analogously by using diethyl sulfate.

(d) *N-N'-Dibenzyl- $\gamma,\gamma'$ -Dipyridylum-Bichloride (or Benzyl Viologen)*.—The benzyl compound is prepared analogously, using benzyl chloride instead of methyl sulfate.  $\gamma,\gamma'$ -Dipyridyl, dissolved in an excess of benzyl chloride, directly yields a precipitate on heating. The boiling is extended to 2 or 3 seconds, the mixture then poured into a large volume of acetone, and a few drops of concentrated hydrochloric acid are added. The precipitate is filtered off and is recrystallized by dissolving in a small amount of methyl alcohol and precipitating with acetone. The preliminary preparation of a picrate is not necessary. If a picrate be prepared it can be converted into the chloride in the same way as with the other picrates. This picrate does not crystallize so easily as the others. Usually it arises first in an amorphous state which on gentle heating gradually becomes crystalline.

(e) *N-N'-Dibetaine- $\gamma,\gamma'$ -Dipyridylum-Dichloride (or Betaine Viologen)*.—The nitrogen of pyridine can be attached to monochloroacetic acid. Then, the chlorine of this compound is present in ionic form and the compound is analogous to the chlorides of a betaine (9). So it could be expected that  $\gamma,\gamma'$ -dipyridyl might



give a double betaine compound. This compound was prepared as follows: 10 gm. monochloroacetic acid are melted in a test-tube and 1 gm.  $\gamma, \gamma'$ -dipyridyl is added. The solution is heated quickly to the boiling point and kept boiling for 2 to 3 seconds. Due to the formation of by-products it turns amber-yellow. The betaine was recovered from this solution in the form of the chloride as the free betaines are very hygroscopic substances. The solution is mixed with a large excess of acetone and several drops of concentrated hydrochloric acid are added. The chloride of the betaine crystallizes from this solution and can be recrystallized by dissolving the dry crystals in a small amount of methanol, adding a large volume of acetone, and, if necessary, reducing the volume on a steam bath until crystallization begins. Further recrystallization may be performed by dissolving with methanol and precipitation with acetone, or benzene.<sup>3</sup>

Analytical data have been presented for the methyl compound in a previous paper (2). The analysis of the benzyl compound, dried at 60° *in vacuo*, is:

3.786 mg. substance gave 9.590 mg. CO<sub>2</sub> and 2.020 mg. water.

3.174 mg. substance gave 8.185 mg. CO<sub>2</sub> and 1.550 mg. water.

7.244 mg. substance gave 0.430 cc. N<sub>2</sub> (20°, 759 mm.).

5.555 mg. substance gave 3.885 mg. AgCl.

Found: C, 69.05, 70.32 per cent; H, 5.97, 5.46 per cent; N, 6.90 per cent; Cl, 17.30 per cent.

Calculated for C<sub>24</sub>H<sub>22</sub>N<sub>2</sub>Cl<sub>2</sub>: C, 70.57 per cent; H, 5.44 per cent; N, 6.86 per cent; Cl, 17.16 per cent.

We are indebted to Dr. H. Elek for the analyses.

### *Peculiarities with Respect to the Technique of the Potentiometric Titration Experiments*

Particular difficulties are involved in the potentiometric titration of these substances, due to a tendency of the potentials to drift. The cause of this drift and its influence upon the interpretation of the results obtained shall first be discussed.

The drift may vary, according to the circumstances, from 0.1 to 2.0 millivolts per minute, and is always in the direction of the potential becoming more positive in time. Such a drift may be due to two causes: either the reduced form of the substance is a labile molecule

<sup>3</sup> It is worth while mentioning that the picrate of the betaine compound cannot be converted to the chloride by the simple method described for the other representatives of this group.

which gradually disappears by an irreversible process, or the nitrogen, bubbling through the electrode vessel, contains oxygen. To begin with the first possibility, it can be shown that the reduced form of any of these substances, in absence of oxygen, is perfectly stable. When a small amount of the oxidized form is dissolved in a suitable buffer, a suitable reductant added, and the tube then sealed, the color developed by reduction does not fade but lasts for weeks. This can be shown as follows: A test-tube with a drawn-out neck is filled with a solution of  $M/10$  sodium carbonate, some glucose, and 0.5 mg., or less, of methyl viologen, and the neck of the tube sealed. The test-tube is kept at  $50^{\circ}\text{C}$ . Gradually the blue color of the reduced form will appear. On shaking, the color will disappear, due to the oxygen of the air bubble remaining in the tube. By repeated shaking and re-reduction at rest, the oxygen will be exhausted, and when this point is reached the color will remain permanently for weeks. Thus it can be proven that the colored substance is not liable to an irreversible destruction during any reasonable period of time, even at pH 10 or 11.

There remains the second explanation that the nitrogen used for the titration experiments contains a trace of oxygen. In all earlier experiments, however, the nitrogen appeared to be free of oxygen. The gas was purified over copper at  $450^{\circ}\text{C}$ . as described previously (10), and the titration vessel was entirely sealed by mercury as described in the same paper. It could be shown also that this nitrogen is satisfactorily purified, by the fact that other dyestuffs showed no drift of the potentials during titration. In order to prove this statement, a series of dyestuffs was selected, which were known to be perfectly stable substances, both in the oxidized and the reduced forms, and yet very sensitive to oxygen in the reduced form. The latter condition will, in general, coincide with the condition that their potential range is very negative. The nitrogen was re-tested with thionine, galloxyanine (11), and rosinduline G G (12). The last dye seemed to be especially suitable on account of its very negative potential range. Sodium hydrosulfite was used as reductant. One experiment will be described in detail. 35 cc. phosphate buffer (pH 7.0), containing  $2.94 \times 10^{-6}$  mol of rosinduline G G, were titrated with hydrosulfite to approximately 50 per cent reduction. The

nitrogen was bubbled continuously at 74.2 cc./min. (4.45 liter/hour). After allowing several minutes for the establishment of the potential, the potential was read at regular intervals. During an observation of 60 minutes there was no drift, not even of 1/10 of a millivolt. Considering the exceptionally strict conditions, *i.e.* the very high dilution of the dye and its very high sensitivity for oxygen, this experiment seems to show a perfect condition of the nitrogen. When, however, the same experiment was performed at pH 11.0, a drift was observed, amounting to 0.2–0.5 mv. per minute. The drift became negligible when the flow of gas was stopped. The interpretation is as follows:

Reid (13) has shown that methylene white is oxidized by molecular oxygen only in a neutral or alkaline medium, whereas at pH 4.0 it is virtually stable in an oxygen atmosphere, unless there is a catalyst, such as a copper salt, present. One may infer from this that the spontaneous oxidizability of the reduced dyes increases with increasing pH. Our nitrogen contained so little oxygen that the rate of oxidation of the reduced rosinduline was practically zero at pH 7.0, but was detectable at pH 11.0. It is understandable that using a gas with a very low oxygen content, the rate of oxidation will be somewhat proportional to the partial pressure of the oxygen. The oxygen pressure being constant, the oxidation may be negligible at pH 7.0, but not so at pH 11.0.

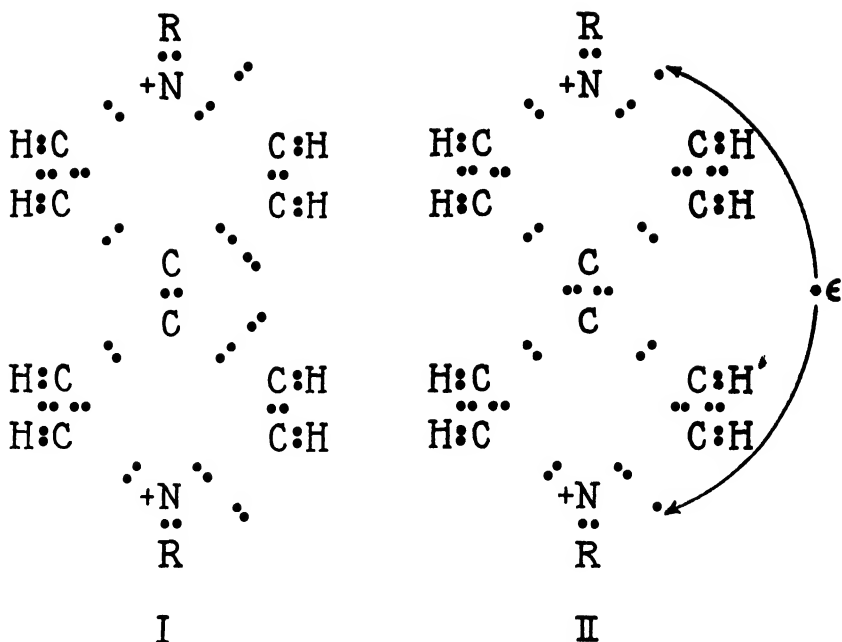
There can be no doubt that any method of purification of nitrogen has its limits. An estimation of the efficiency of the method used for these experiments may be obtained as follows: According to von Wartenberg (14), the cloudy halo that surrounds a piece of yellow phosphorus is still visible in an atmosphere containing 2 parts of oxygen in 100,000 parts of an indifferent gas but not visible in an atmosphere containing 1 part in 100,000. Our nitrogen caused the halo around phosphorus to disappear, so we may estimate the oxygen content of our nitrogen to be 1 in 100,000, or less. If it were only half this amount, it would be sufficient to cause a slight drift in the potential, provided the rate of the effect of this oxygen is appreciable. Experience suggests that this effect is unappreciable at pH 7.0 but is quite detectable at pH 11.0.

On titrating the viologens, we are faced with a situation where we not only have to deal with exceptionally oxygen-sensitive substances, but also are compelled to titrate at, or very near to, pH 11.0. The potential range of these substances, even at pH 11.0 or 10.0, is so close to the hydrogen potential at the same pH that full titration curves cannot be obtained in less alkaline solutions.

Therefore, we are justified in attributing the drifts to traces of oxygen and must be satisfied with a smaller degree of accuracy than might be desirable, but one has to bear in mind that on performing the titration not too slowly, the whole drift during the course of the titration will only amount to 2 or 3 mv. This will cause only a slight distortion of the titration curve and an uncertainty of the normal potentials of only a few millivolts.

*Interpretation of the Potentiometric Curves and Suggestions as to the Chemical Structure of the Compounds*

On taking  $\pm 2-3$  mv. as limits of error, and applying the principles developed previously (15), we may state that the shape of the titration curve is that of a one-electron system and that the normal potential is independent of pH throughout that range in which a full titration curve can be obtained; *i.e.*, pH 10.0–13.0. Both the oxidized and the reduced forms have the character of a quaternary base and exist in a strongly, if not completely, ionized state at any pH, without changing the state of dissociation with pH. So the two forms differ from each other only by an electron and never by a full hydrogen atom (or a hydroxyl group) and the normal potential must be considered as independent of pH, not only for that range of pH in which titration experiments can be executed, but also in acid solution, in which, on account of the overvoltage, such experiments cannot be performed. The following formula will give a picture of the structure.



The bivalent cation of the (colorless) bi-quaternary ammonium base.  $R$  means a univalent radical such as  $\text{CH}_3$ . The pyridine rings have the benzenoid structure.

The univalent cation of the (violet) reduced form. One electron (designated as  $\epsilon$ ) has been added to formula I and the double bonds have been rearranged. The odd electron  $\epsilon$  is shared by both N atoms to supplement the septet to an octet, alternately, in rapid succession; this electron belongs to the one and the other N atom. The pyridine rings have the quinoid structure.

The molecule II can be reduced farther. The next step of reduction is the attachment of another electron to formula II. Then the structure of the right hand formula is in general maintained, except for the fact that the octet of either N atom is permanently complete without any sharing of an odd electron being necessary. These compounds are almost colorless, slightly yellow. One representative, namely the benzyl derivative, can be easily produced by reducing benzyl viologen in a very alkaline solution ( $\text{pH} > 12$ ) by an excess of hydrosulfite or by glucose beyond the violet stage. On exposure to the air, oxidation to the violet form and subsequent oxidation to the colorless viologen takes place. This, however, is true only for the freshly prepared solution; an irreversible destruction of this molecule gradually takes place. This substance is on the reduction level of a dihydroadipiridyl. Still further reduction leads to the level of the tetrahydroadipiridyl, of which the substance produced by reduction of pyridine by zinc in the presence of acetic anhydride is an example. No substance on the trihydroadipiridyl level has been known.

*Potentiometric Titrations*

Potentiometric titrations were performed for the aqueous solution with sodium hydrosulfite at 30°C. The most convenient buffer is made from disodium phosphate and sodium hydroxide, but the titrations can be performed at any pH between 13.0 and 10.0, or even 9.0. At lower pH the titration curves are incomplete because of the overlapping with the hydrogen potential. The completeness of the titration curve is indicated by a jump of the potential into a more negative level at the end.

The following tables are examples of the titrations.

*Methyl Viologen*

pH = 11.0 ( $\text{Na}_2\text{HPO}_4 + \text{NaOH}$ ).

Titrated with sodium hydrosulfite at 30°C.  $\pm 0.05$ .

Potentials calculated according to the formula

$$E = -0.446 - 0.0601 \log \frac{\text{Per cent reduction}}{100 - \text{per cent reduction}}$$

All potentials referred to the normal hydrogen electrode at 30°C.

Reduction	Observed potential	Calculated potential
<i>per cent</i>		
0.0		
13.4	0.3977	0.3986
19.6	0.4102	0.4107
30.5	0.4252	0.4254
39.8	0.4355	0.4360
49.0	0.4449	0.4450
57.5	0.4531	0.4538
67.7	0.4634	0.4652
77.0	0.4740	0.4774
89.5	0.4940	0.5075

Similar results were obtained in many other experiments, in which the initial amount of the substance was varied from  $4 \times 10^{-7}$  mols up to  $4 \times 10^{-6}$  mols, dissolved in 30 cc. of the buffer; furthermore in pH range varying from 9 to 13.

### *Ethyl Viologen*

pH = 11.0 (Na<sub>2</sub>HPO<sub>4</sub> + NaOH).

$$E = -0.449 - 0.0601 \log \frac{\text{Per cent reduction}}{100 - \text{per cent reduction}}$$

Reduction	Observed potential	Calculated potential
<i>per cent</i>		
0.0		
7.73	-0.3830	-0.3844
15.4	-0.4050	-0.4053
22.6	-0.4165	-0.4170
35.1	-0.4326	-0.4330
44.6	-0.4431	-0.4434
60.1	-0.4595	-0.4596
74.4	-0.4750	-0.4767
92.8	-0.5120	-0.5122

### *Betaine Viologen*

pH = 11.0 (Na<sub>2</sub>HPO<sub>4</sub> + NaOH).

$$E = -0.444 - 0.0601 \log \frac{\text{Per cent reduction}}{100 - \text{per cent reduction}}$$

Reduction	Observed potential	Calculated potential
<i>per cent</i>		
0.0		
7.10	-0.3770	-0.3770
14.4	-0.3980	-0.3984
21.0	-0.4103	-0.4105
30.6	-0.4225	-0.4233
39.4	-0.4331	-0.4332
53.4	-0.4472	-0.4475
71.5	-0.4672	-0.4679
96.8	-0.5235	-0.5320

### *Benzyl Viologen*

pH = 8.0 (Na<sub>2</sub>HPO<sub>4</sub> + KH<sub>2</sub>PO<sub>4</sub>).

$$E = -0.359 - 0.0601 \log \frac{\text{Per cent reduction}}{100 - \text{per cent reduction}}$$

Reduction	Observed potential	Calculated potential
<i>per cent</i>		
13.1	-0.3087	-0.3097
18.0	-0.3203	-0.3205
22.5	-0.3279	-0.3272
28.0	-0.3353	-0.3356
33.6	-0.3420	-0.3427
39.6	-0.3487	-0.3480
46.0	-0.3555	-0.3554
54.1	-0.3645	-0.3648
63.3	-0.3773	-0.3772
71.1	-0.3924	-0.3924
80.6	-0.3961	-0.4145
91.6	-0.4070	-0.4215

*Absorption Spectra of the Dyes*

The absorption spectra of all these indicators, in their reduced form, consist of a very distinct band. Within the band one can distinguish

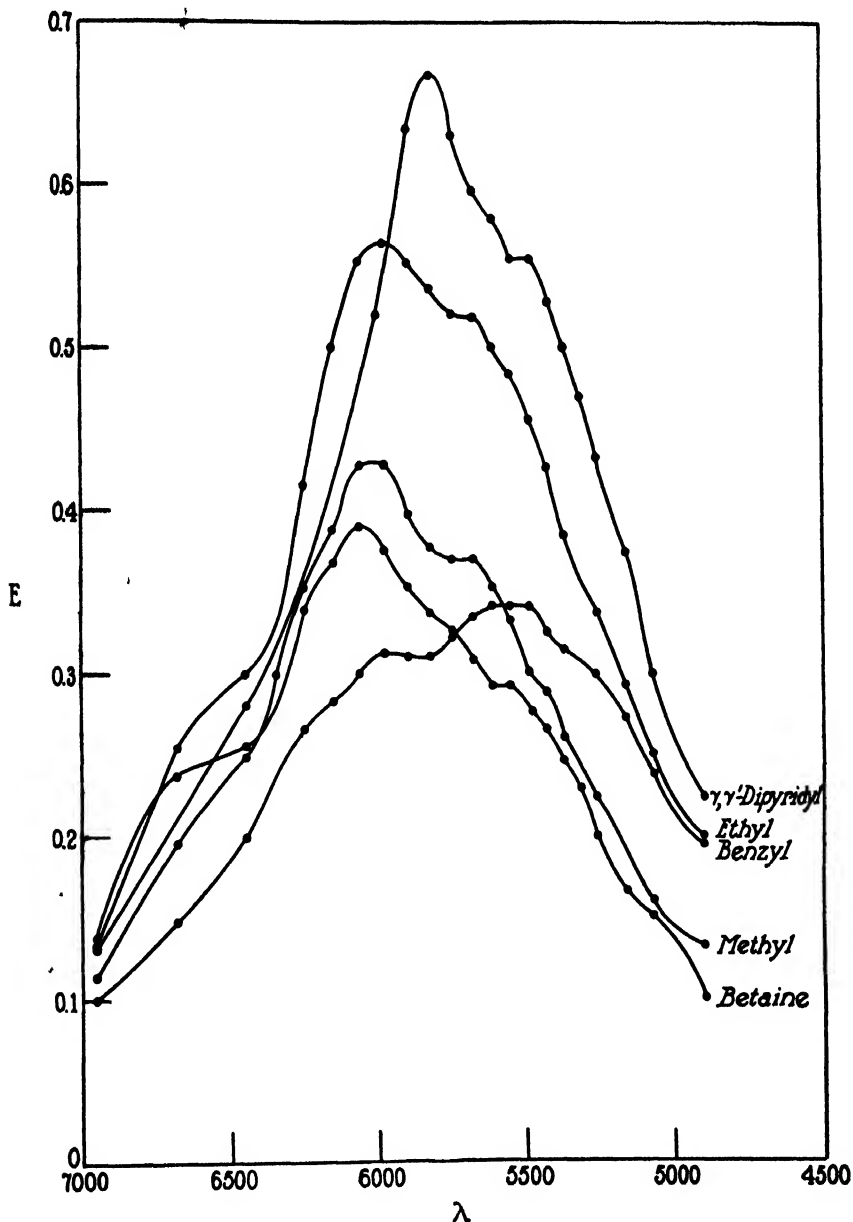


FIG. 1. Extinction,  $E$ , plotted against wave length,  $\lambda$ .  $E$  is the logarithm of the ratio of the intensity of the incident light to the transmitted light.



one distinct maximum of absorption and a noticeable secondary maximum. The primary peak of absorption is the one towards the longer wave length side, except for the benzyl compound, where the converse is true. Beer's law holds for these dyes within wide limits. The absorption for a highly concentrated solution in a very thin layer matches that of a solution more than 20-fold weaker in a correspondingly thicker layer. The spectrophotometric measurements were made with the spectrophotometer according to König and Martens in a solution of the substances kept in the reduced state by adding glucose in a phosphate buffer (pH 10.0-11.0). The curves in Fig. 1 show the extinction plotted against wave lengths. The concentration of the dye is different for each curve in order to allow the plotting on one graph. Therefore the absolute value of the ordinate of one curve must not be compared with that of any other. But the general shape and especially the location of the peaks are comparable. For comparison, the spectrum of the reduction product of  $\gamma, \gamma'$ -dipyridyl as produced by reducing this substance in dilute acetic acid with chromous chloride has been plotted. Because of the instability of this color over a longer period of time the accuracy of this particular curve as to details may not be very high, but good enough to determine the maximum and the secondary peak of the absorption. The location of the various maxima is as follows:

	First peak	Second peak
	$m\mu$	$m\mu$
$\gamma, \gamma'$ -Dipyridyl.....	582	550
Methyl viologen.....	601	570
Ethyl viologen.....	598	570
Betaine viologen.....	605	558
Benzyl viologen.....	598	555

Smaller than  
the first  
peak  
Higher than  
the first

The color of the reduced methyl, ethyl, and betaine viologen is indigo blue in very high dilution, somewhat more violet in more concentrated solution; that of the benzyl compound is violet in very high dilution and more blue in higher concentration. The reduced form of  $\gamma, \gamma'$ -dipyridyl itself is more red-violet.

The above table does not account for the smaller secondary maxima

which are especially obvious in the betaine compound. A remarkable peculiarity of benzyl viologen is the dependence of its absorption spectrum on the temperature. The color of the reduced form varies, in a reversible way, from reddish-violet at room temperature, to a pure blue at high temperatures. In the other compounds a small trace of this phenomenon is just detectable: the color changes reversibly with increasing temperature from violet-blue to a purer blue. The spectroscopic observation shows that the change with increasing temperature in the benzyl compound is due to a decrease of the first maximum and an increase of the second maximum of absorption. At 80° the first maximum is very high, and the second becomes inconspicuous so that the type of the spectrum becomes the same as for the other compounds, whereas at low temperature it differs greatly. For the reduced form of  $\gamma, \gamma'$ -dipyridyl itself no change with temperature is noticeable.

#### *The Application of the Substances as Indicators*

The determination of the oxidation-reduction potentials, within suitable ranges, by means of these indicators can be made by comparing the color intensity produced by the solution in question with the color intensity which could be produced by the complete reduction of the same amount of indicator. The problem is therefore only to establish a standard solution in which the indicator may be considered as completely reduced. The difficulty in preparing such a standard solution lies in the fact that by applying too strong a reductant the reduction may go beyond the first step and partially destroy the color. A complete reduction without the risk of overreduction can be brought about by means of a solution of 2-5 per cent glucose in  $M/10$   $Na_2CO_3$ , except for benzyl viologen. Here, at  $pH \geq 10.0$ , gradually an overreduction would take place. The reduction, however, will be complete without overreduction at  $pH$  about 9.8; *i.e.*, a mixture of 6 parts of  $M/5$  sodium carbonate plus 4 parts of  $M/5$  sodium bicarbonate.

#### *The Problem of Hydrogen Overvoltage*

The problem in question can be best explained by an example. Supposing we wish to reduce methyl viologen in an acid solution. We

can do so by using chromous chloride as a reductant. The success of the reduction becomes evident by the appearance of the deep blue color. The chromous-chromic-ion system is supposed to have a normal potential of about  $-0.4$  volt, which at least in all ranges of pH will not depend appreciably on pH. So it is understandable that chromous chloride in an acid solution (pH 2 to 4) containing no, or only little, chromic chloride, will reduce to a certain extent viologen, the normal potential of which is about  $-0.4$  volt. The striking fact is that such an overvoltage potential can be maintained in an aqueous medium. One might expect that under the conditions of such an experiment water would be reduced and hydrogen gas be developed rather than that viologen would be reduced. When colloidal palladium is added to the system viologen + chromous chloride, the blue color of the reduced viologen will not arise and the expected development of hydrogen gas within the solution will take place instead. The only interpretation imaginable is the hypothesis that the primary reduction product of water is not  $H_2$ , but monomolecular hydrogen. In the absence of a catalyst the hydrogen atoms will be accumulated until equilibrium is attained. When, however, a catalyst is present which brings about the reaction  $2H \rightleftharpoons H_2$  with appreciable speed, the overvoltage potential will break down.

In the absence of a catalyst these indicators can be used even for the measurements of potentials in the hydrogen overvoltage ranges. In this respect the potential measurement by means of such an indicator is far superior to a measurement by metal electrodes. Platinum or gold electrodes, even when blank, will allow only a very small overvoltage. Only mercury electrodes will stand an appreciable overvoltage, and for this reason mercury electrodes were used, e.g. by Forbes and Richter (16), for measurement of the potential of chromous-chromic systems. The application of this mercury electrode measurement in an overvoltage range meets with great technical difficulties. There is scarcely any difficulty in applying the indicator method.

#### SUMMARY

The tabulation gives the normal potentials of the various indicators at  $30^\circ C.$ ; referred to the normal hydrogen electrode, the accuracy is estimated to be  $\pm 0.002$  volt.

## Normal potentials of the viologens at 30°C.:

Methyl viologen	-0.446 volts
Ethyl viologen	-0.449 volts
Betaine viologen	-0.444 volts
Benzyl viologen	-0.359 volts

Supposing some solution brings about a coloration of one of these indicators to the extent of  $A$  per cent of the maximum color, the oxidation-reduction potential of this solution is  $E = E_o - 0.06 \log \frac{A}{100 - A}$  where  $E_o$  is the normal potential according to the above tabulation. This normal potential is independent of pH.

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# MOVEMENT IN THE CYANOPHYCEAE

## THE EFFECT OF pH UPON MOVEMENT IN *OSCILLATORIA*

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As part of a comprehensive study of the nature and mechanism of the movements of Cyanophyceae, the effect of hydrogen ion concentration upon the velocity of translatory movement in *Oscillatoria formosa* Bory was determined under controlled conditions. The profound influence of the hydrogen ion upon ameboid movement has been demonstrated (Pantin, 1923), and the effect of pH upon various metabolic processes of motile autotrophic organisms has been shown by a number of investigators. Among others, Maertens (1914) has reported that *Oscillatoria* grows best at slightly alkaline reactions in suitable culture media. The lethal effect of acids upon *Oscillatoria* was noted by Schmid (1923), and a negative chemotactic response was observed by Fechner (1915). However, no quantitative information concerning the speed of movement of this alga in relation to hydrogen ion concentration has been found in the literature.

### Methods

*Oscillatoria* was collected from the mud flats along the Charles River (Cambridge, Mass.) and grown on 1 per cent nutrient agar impregnated with inorganic salts according to a formula employed by Uspensky and Uspenskaja (1925). The stock cultures were isolated from a single filament and grown in Petri dishes under diffuse daylight.

For experimental purposes, the following procedure was adopted: the dishes were wrapped in clear cellophane to conserve the moisture content of the agar and placed in a constant temperature incubator (about 18°C.) some 30 cm. distant from a 25 watt frosted bulb. After a minimum period of 15 hours' adaptation to these conditions, the alga was deemed ready for transfer to the observation cells. The cells employed were of the Van Tieghem type, each consisting of a glass ring

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\* National Research Council Fellow in the Biological Sciences.

cemented with Picein to a microscopic slide and a round glass cover-lid. After carefully greasing the upper rims with vaseline, the cells were filled with the stock culture solution. Small bits of agar containing the *Oscillatoria* filaments were transferred with a platinum needle to a series of the cover-lids wet with drops of the same culture solution. Then the inoculated covers were dropped carefully upon their respective cells and sealed without the formation of air bubbles. The series of preparations was then placed in an inverted position in the incubator for a period of about 5 hours, until the algal filaments had begun to glide freely over the inner surface of the cover-glass. Different experimental solutions could be substituted in the cells by merely sliding the covers aside sufficiently to permit entrance of a fine pipette. The filaments adhering to the glass formed a suitable preparation for microscopical examination.

The source of illumination for the microscopical observations consisted of a 100 watt tungsten ribbon filament bulb focused with a condensing lens onto the plane substage mirror. From the mirror the light was reflected upward to a finely ground glass plate placed just beneath the stage, and thence directly illuminated the cell containing the organisms. An intensity of 26 foot candles was used (measured with a Weston photronic cell placed on the microscope stage). Infra-red radiation was effectively removed (Coblentz, 1911) by interposing between the light source and the microscope a glass cooling cell containing a 2.2 cm. thickness of 2.5 per cent solution of copper chloride in water.

All observations were made at 22°C. with the microscope placed in a constant temperature box which could be kept within a variation of  $\pm 0.1^\circ\text{C}$ . The time required for a filament to traverse one space (16.6 microns) of the ocular micrometer was measured with a stop-watch. From the average of two readings for a filament, the velocity was computed in terms of microns per second. Ten different filaments, which appeared free to move in an unimpeded manner, were chosen for each given set of readings. A set of observations could usually be made in 10 to 15 minutes, depending upon the speed of the *Oscillatoria* as well as upon the dexterity of the observer. The fact that repeated observations at intervals could not be performed upon the same filaments is not considered a serious objection, since similar methods of observation upon *Oscillatoria* have hitherto given clear results (cf. Crozier and Federighi, 1924).

The solutions were always made up in large volume with glass-distilled water; aliquot parts were adjusted to the desired pH with acid or base. The lower pH values were determined electrometrically with the quinhydrone electrode and in the solutions above pH 7.3 with the hydrogen electrode.<sup>1</sup>

A series of solutions ranging from pH 4.2 to 11.2 was obtained by the addition of N/10 HCl or N/10 NaOH to 100 cc. portions of Uspensky's solution modified to include  $\text{K}_2\text{HPO}_4$ . The pH of this stock

<sup>1</sup> Thanks are due Dr. P. S. Tang for his kind assistance with the hydrogen electrode determinations.

solution, as made up, was 7.05; the concentration of salts was 0.211 gm. per liter. From two to six separate cells were set up for each pH value at different times and a set of readings was made on ten filaments in each cell, starting 5 minutes after initial immersion of the organisms in the test solutions. At the end of an hour, during which time the cell remained in the light on the microscope stage, another set of

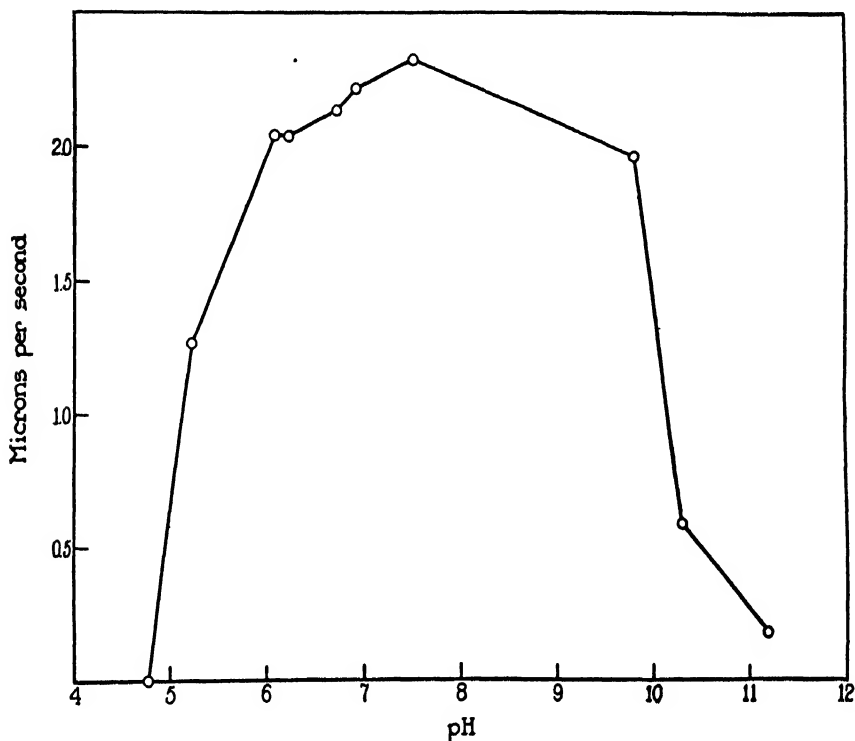


FIG. 1. Mean rates of translatory movement of *Oscillatoria* after immersion for 1 hour in solutions at different pH values. The number of filaments timed were: 20 at pH 4.75 and 11.2; 30 at pH 6.05, 9.8, and 10.3; 50 at pH 5.2 and 7.5; and 60 at pH 6.2 and 6.7.

readings was made. Thus a total of 1600 readings were made upon forty different cells in the course of this experiment.

The means of the observations at the beginning and end of the hour showed close agreement for each pH value, with indications of progressive inhibition in the extreme pH ranges. In Fig. 1 there are plotted the mean rates of all readings taken at each pH after approx-



imately 1 hour. Fair activity is indicated between pH 6.0 and 9.8, but beyond these points the decrease in velocity is striking. A wide favorable range appears to lie on the basic side of neutrality.

Since this solution contained carbonates and relatively high ratios of Ca and Mg which precipitated out at the higher alkaline reactions, a similar experiment was performed using a different type of formula in

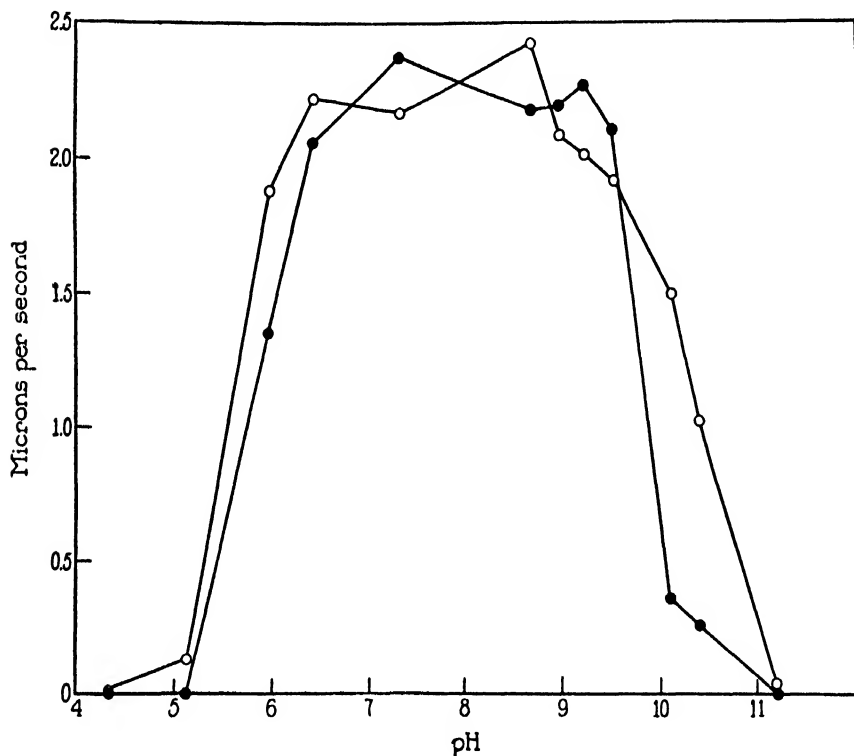


FIG. 2. Mean rates of translatory movement of *Oscillatoria* after immersion for 2 hours (open circles) and for 8 to 10 hours (solid circles) in solutions of different pH. Each point represents the mean value for ten filaments.

making up the stock solution. This experiment was planned to test the influence of the time factor upon movement in solutions covering a wide pH range. A modification of the solution employed by Maertens (1914) in his investigation of the optimum  $H^+$  ion concentration for the growth of blue-green algae was utilized as a stock. The composition of the solution was as follows: Pyrex-distilled water—1000 cc.;  $KNO_3$ —0.1 gm.;  $MgSO_4$ —0.05 gm.;  $K_2HPO_4$ —0.2 gm.;

$\text{Ca}(\text{NO}_3)_2$ —0.04 gm.; and  $\text{Fe}_2(\text{SO}_4)_3$ —0.0007 gm. (total salt content of 0.3907 gm. per liter). The pH of this solution, as made up, was 7.3; from it a series of thirteen different solutions was adjusted with N/10 HCl or N/10 KOH to cover the range pH 4.35 to 11.2. No precipitate could be seen in the alkaline solutions and the maximum amount of acid or base required to obtain the extreme ranges was

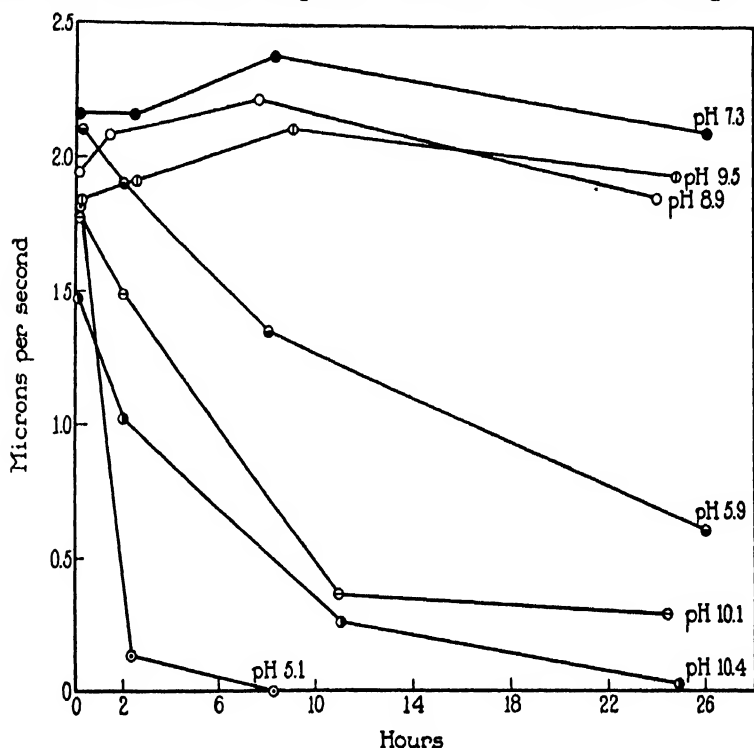


FIG. 3. Mean rates of translatory movement of *Oscillatoria* at various time intervals after immersion in solutions of different pH. Each point represents the mean value for ten filaments.

considered negligible from the viewpoint of osmotic effects of the total salt concentration.

Movement of the *Oscillatoria* was observed at repeated intervals after immersion in the respective pH solutions; *i.e.*, at approximately 5 to 15 minutes, 2 hours, 8 to 10 hours, and 24 to 26 hours. During the intervals between readings the cells were kept in the incubator and at from 10 to 15 minutes preceding each of the repeated sets of observations fresh solutions were placed in the cells. Several sets of

data were procured from different cultures, and though the pH range showed close agreement in all, the actual rates varied from one culture to another, probably due to varying nutrient relations or different water content of the agar. The results of one series of observations for a single culture are presented in Figs. 2 and 3.

The data show favorable conditions for movement in a relatively wide alkaline range up to about pH 9.5. Below neutrality, activity appears to be checked in the region of pH 6.4. Furthermore, at unfavorable pH values the inhibition is progressive with exposure time. That this inhibition is due to pH, and not to the method of handling nor to improper adjustment of the dissolved gases, is supported by the continuous high rates for 24 hours in the favorable alkaline range mentioned.

It was noted that in acid solutions where inhibition was complete there occurred a shrinkage of the protoplasts followed by dissolution of the filaments. In the inhibiting alkaline solutions there was on the contrary a suggestion of swelling in the vicinity of the transverse walls, in such a way as to cause separation of the apical cells by a wide hyaline area.

It appears that movement occurs within the pH range which has been indicated as best for the growth of these organisms. The actual velocity of locomotion as well as the pH range agrees well with the data reported for ameboid progression in contact with glass. Of course the effect of the hydrogen ion would be modified, were other factors altered; *i.e.*, the salt content, temperature, light, kind of substratum, etc. The effect of pH in relation to other variables influencing motility demands further investigation.

#### SUMMARY

The effect of pH upon the velocity of translatory movement of *Oscillatoria formosa* Bory in inorganic culture solutions was determined.

Unhindered movement occurred in the range of about pH 6.4 to 9.5. Above and below these limits inhibition was marked.

In the unfavorable acid and alkaline ranges inhibition was progressive with exposure time; in the favorable range continuous movement was maintained for 24 hours.

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# ANALYSIS OF THE GEOTROPIC ORIENTATION OF YOUNG RATS. VIII

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## I

Characteristic differences have been demonstrated among the curves representing for each of a number of well-inbred strains of rats the relation between the angle ( $\theta$ ) of upward, geotropically oriented progression upon an inclined surface, and the slope of the surface ( $\alpha$ ). For a given race, at standard age (13 to 14 days), this curve is a reproducible property (Crozier and Pincus, 1931-32*a*). Adequate indications have been obtained as to the genetic character of the differences which serve to contrast diverse races in this respect (Crozier and Pincus, 1929-30*a* and *b*; 1931-32*c*). The *variation* of performance (*i.e.*, of  $\theta$ ) is also a characteristic property (Crozier and Pincus, 1929-30*a*; 1931-32*a*); at equal magnitudes of performance it is not affected by the carrying of additional loads such as modify the speed of creeping and the magnitude of the gravitational pull experienced and thus influence the extent of the oriented response (Crozier and Pincus, 1931-32*b, c*). The total *amount* of variation of  $\theta$  may be influenced, as by the injection of adrenin, but the proportion of this total which is susceptible to control as a function of the slope of the surface seems not to be affected (Crozier and Pincus, 1932-33).

It is important to determine whether these characteristic differences among the strains of rats examined are retained during growth, or are instead dependent upon age. Suitably prepared *adult* rats exhibit upward geotropic orientation such that  $\theta$  continues to be a definite function of slope ( $\alpha$ ) of surface (Upton and Stavsky, 1932; Crozier, Pincus, and Stavsky, in press). The inquiry has two immediate aspects: Are the curves ( $\theta$  vs.  $\alpha$ ) for the several races typically diverse when the performance of adult rats is measured? And, do the indices

of variation, that is, the measures of *variability* (Crozier, 1929), change with age or with experimental treatments?

With young rats, of race *A*, total variation of performance is increased after intraperitoneal injection of adrenin, although the fraction of the total which is controlled by the intensity of excitation is unaltered (Crozier and Pincus, 1931-32 *a*); since the relative variation of performance has been correlated (Crozier and Pincus, 1931-32 *c*) with the total number of functioning sensory units involved in the excitation by gravitational pull, it should be possible to test the notion that the "total number of sensory units" may be a characteristic feature of the race (genotype), independent of age. The consideration of  $\theta$  permits comparisons among results under conditions such that the  $\theta$  vs.  $\alpha$  curve is markedly altered. The *a priori* reasons for expecting that  $\sigma/\theta$  should be a function of  $\theta$  have been considered previously (Crozier and Pincus, 1926-27). In the present case we desire to deal with the alteration of the  $\theta$ - $\alpha$  curve which may be induced in the performance of adult rats of race *A* following injection of adrenin. With young rats so treated with an appropriate dosage (1) the threshold slope of surface for the evocation of gravitationally oriented creeping is lowered, and (2) the extent of upward orientation on surfaces of higher slopes is increased, except at the very highest usable inclinations. If the indices of variability are unaffected by age or by experimental modification of the  $\theta$ - $\alpha$  graph, potent evidence is given that the capacity for variation of performance under these conditions is a genetic invariant.

## II

Adult rats of race *A* were prepared for observation by being fed after creeping upward upon an inclined runway (*cf.* Upton and Stavsky, 1932). The inclination ( $\alpha$ ) of the runway was varied irregularly during the period of preparation, 12 to 14 days in duration; two feeding intervals were used on each day. The only detectable outcome of the procedure is suppression of the effects which in the case of an unprepared adult rat allow it to creep at random (although very rarely indeed downward).

\*The two series of experiments here discussed were carried through after considerable experience with other groups of adult rats. Adrenalin chloride 1:25,000 in Ringer solution to 1:100,000 was injected

TABLE I

Mean orientation angles ( $\theta$ ) for adult rats, race *A*, at various slopes of surface ( $\alpha$ );  $N = 4$  individuals,  $n = 45$  observations, at each slope.

Order of observation	$\alpha$	$\theta_m$	P.E. $_{\theta_m}$
	<i>degrees</i>	<i>degrees</i>	<i>degrees</i>
7	15	59.04	$\pm 1.23$
1	20	62.20	1.48
10	25	62.80	1.45
2	30	63.56	1.25
16		63.93	0.955
12	35	66.06	1.07
3	40	67.79	0.964
8	45	70.98	0.957
4	55	73.49	0.837
15		77.14	0.709
9	60	78.68	0.714
13		77.81	0.711
5	70	78.07	0.781
14		81.53	0.482
11	75	80.44	0.781
6	80	82.94	0.518

TABLE II

Mean orientation angles ( $\theta_m$ ) for adult rats of *A* strain, following intraperitoneal injection of 0.5 cc. of adrenalin chloride 1:100,000; trails taken  $\frac{1}{2}$  hour to 1 hour subsequent to injection.  $N = 4$  individuals,  $n = 45$  observations, at each slope.

$\alpha$	$\theta_m$	P.E. $_{\theta_m}$
<i>degrees</i>	<i>degrees</i>	<i>degrees</i>
12.5	58.94	$\pm 1.40$
15	62.05	1.25
20	64.00	1.05
30	67.63	0.997
40	72.35	1.08
55	77.30	0.694
65	81.30	0.587
80	82.67	0.600

intraperitoneally, 0.5 ml. being injected  $\frac{1}{2}$  hour to 1 hour before the recording of trails made upon the inclined plane (Crozier and Pincus, 1931-32*a*). The temperature was 20-22°C. throughout. As with



the young *A* rats (Crozier and Pincus, 1932-33), spontaneous pauses in creeping are fewer and of shorter duration following the injection with adrenin, and progression is considerably accelerated.

Data upon the orientation of uninjected adult *A* rats (Series I) are given in Table I. The two determinations obtained at  $\alpha = 30^\circ$ , one at the beginning and one at the close of the observations, show by

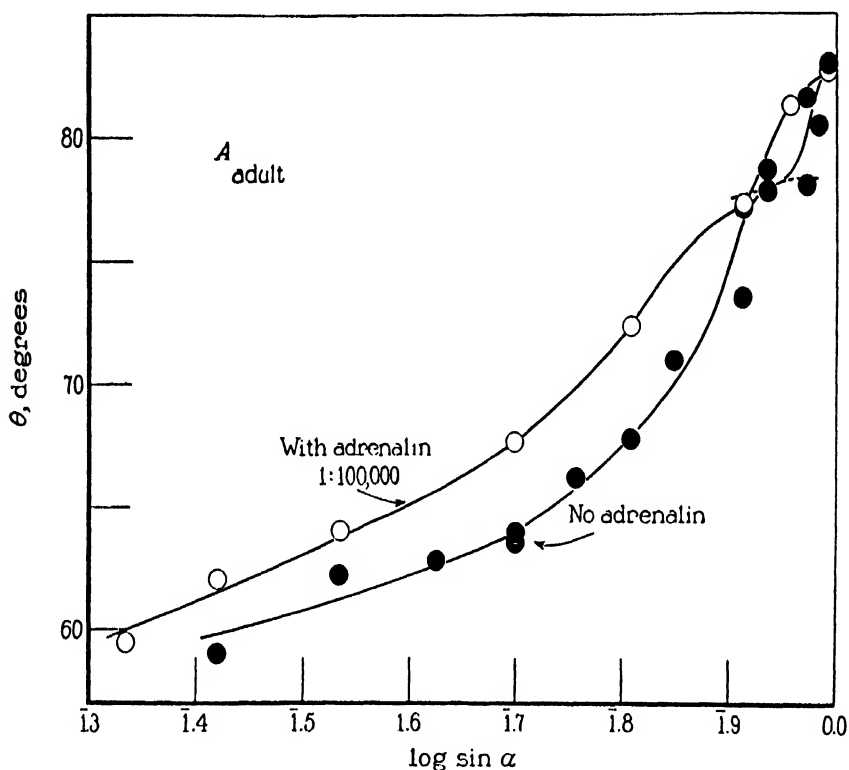


FIG. 1. Mean orientation angles ( $\theta$ ) for adult rats of race *A*, Series I;  $N = 4$  (2 ♀ ♀, 2 ♂ ♂),  $n = 45$ ; data in Tables I and II.

their close agreement that there is no progressive "training" effect during the measurements; this has also been tested thoroughly in other sets of observations. These figures are plotted in Fig. 1.

When injected with adrenalin 1:100,000, 0.5 ml., intraperitoneally, these individuals gave mean orientation angles as listed in Table II, also plotted in Fig. 1.

## III

The curve of  $\theta$  vs.  $\alpha$  for the uninjected adult *A* rats is quantitatively different from that for adult *K* rats (*cf.* Crozier and Pincus, 1931-32 *a*; Upton and Stavsky, 1932; Crozier, Pincus, and Stavsky, in press), but is of the same general nature so far as concerns its contrasts with the corresponding curve for the young individuals of the same strain

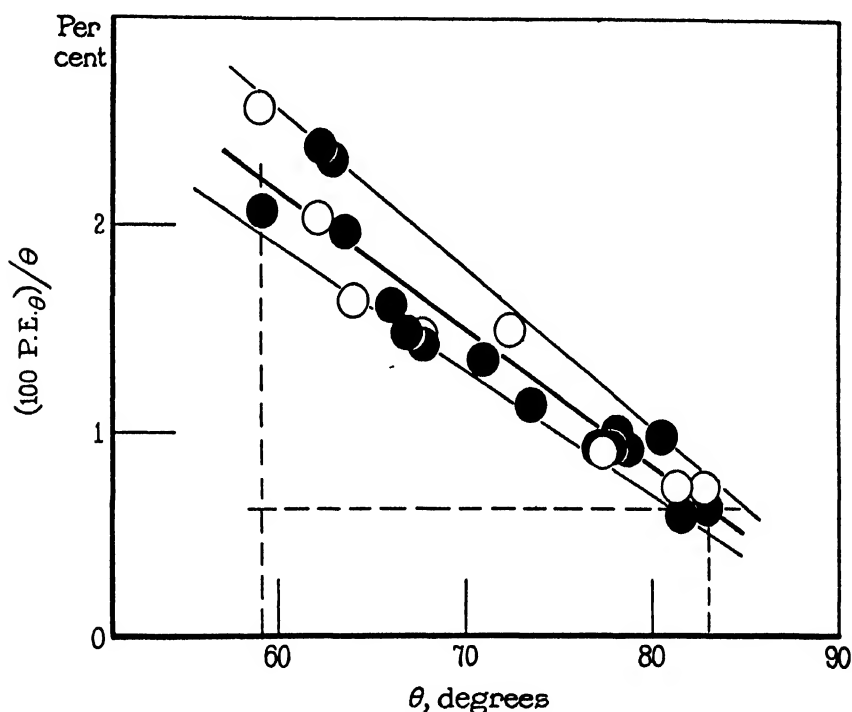


FIG. 2. The relative variation of orientation is the same for adults of race *A*, at equal magnitudes of performance, with adrenin injection (open circlets) or without.

(Crozier and Pincus, 1931-32 *a*). The threshold  $\alpha$  is lower, and the curve's shape is different. The characteristic "break" near its upper end is present. This, as in the case of the curve for young guinea pigs (*cf.* Upton, 1931-32), is due in the adult rats to the incidence of a different mode of progression, namely galloping, which involves a different disposition of gravitationally induced pulls upon the legs as compared with the creeping on surfaces of lower slopes. As with the young

*A*'s, the faster creeping subsequent to injection of adrenin brings about orientation at a lower  $\alpha$ , and a general sideward displacement of the  $\theta$ - $\alpha$  curve. At the new threshold slope of surface, however, the threshold response ( $\theta$ ) is unaffected.

We are specifically interested in the variation of performance. As shown in Fig. 2,  $P.E._\theta/\theta$  is a declining rectilinear function of  $\theta$ , as with various assemblages of young rats (Crozier and Pincus, 1929-30*b*; 1931-32*c*). The total observed variation (the area under the graph) is 95.8 units, corrected for  $N$  and for  $n$  (Crozier, 1929; Crozier and Pincus, 1931-32*b*). This is slightly higher than with the young rats (86 units). But the fraction of this total which is modified as a function of  $\theta$  is 56.1 per cent. This is identical with the value established for the young *A* individuals (Crozier and Pincus, 1931-32*d*), with adrenin or without.

The variation data for  $\theta$  from the rats injected with adrenin are also given in Fig. 2.  $N$  and  $n$  are the same for both sets, so the two series are directly comparable. Within limits set by the fact that each  $P.E._\theta$  is subject to a standard deviation proportional to its own magnitude, the indices of variation with and without adrenin are identical. In this respect the effect of adrenin upon the variation of orientation angle differs from that observed in the young *A* rats. There, the *total* variation was markedly enhanced (86 units normally to 127 units with adrenin), although the fraction modifiable was unaffected. It is as if factors connected with the ordinary orienting performance of the adults were a little more complex than with the young; this might be merely a consequence of faster progression and speedier orientation, or of the different posture of the legs in the adults. There is a slight and probably quite non-significant decrease of variance of  $\theta$ , at constant  $\alpha$ , with the progress of a succession of tests in the case of adults (*cf.* Table I, *e.g.*) which might be significant in this connection if real. The action of adrenin in the young *A* rats is to make the orientation process, at given  $\theta$ , a little less sure and certain, the modifiable and the unmodifiable moieties being equally affected, as might be the case if there were a slight and more or less irregular increase in muscular tonus in the legs. This is not at all apparent in the data for adults in Table I.

The variation here dealt with differs from that apparent in (young)

hybrid rats (Crozier and Pincus, 1931-32*e*), where very complex (but analysable) effects are in evidence. We do not as yet know whether the peculiarities of our hybrid rats are continued into their adulthood; and it will be peculiarly interesting to see whether the increased variation of  $\theta$  which they show, and their lower proportionate modifiable variation, will respond to the injection of adrenalin in the fashion here disclosed.

## IV

Since the dosage with adrenin was rather low in Series I, and since we desired to see if very marked increase in the dosage might not increase the general variation of  $\theta$ , a second set of experiments was made

TABLE III

The mean orientation angles ( $\theta$ ) for adult rats, race A (2 ♀ ♀, 2 ♂ ♂), normal and injected with adrenin.

$\alpha$	Uninjected	$\theta$ Adrenin 1:50,000	$\theta$ Adrenin 1:25,000
<i>degrees</i>		<i>degrees</i>	<i>degrees</i>
20	60.46 $\pm$ 1.45		
25	61.22 $\pm$ 1.24	66.58 $\pm$ 1.07	69.30 $\pm$ 1.23
35	66.21 $\pm$ 1.33	71.35 $\pm$ 1.14	73.80 $\pm$ 0.869
45	74.30 $\pm$ 0.857	76.04 $\pm$ 1.05	78.46 $\pm$ 0.740
55	78.54 $\pm$ 0.716		

upon a slightly different plan. After preliminary establishment of several points upon a normal  $\theta$  vs.  $\alpha$  plot for another lot of four rats, tests were carried out after injection of 0.5 ml. adrenalin chloride at dilutions 1:50,000 and 1:25,000. The results are collected in Table III. The curves in Fig. 3 demonstrate that the effect of adrenin upon the  $\theta$  vs.  $\alpha$  graph is a quantitative one,  $\theta$  being increased at constant  $\alpha$  as a function of the dosage.

The speed of creeping after the maximum dosage used is about twice that in the absence of adrenin, and heavier dosages seemed impracticable to use. The increased speed of progression is evident on the inclined surface, not when the rats are on a level surface, and is thus in the nature of a response to gravitationally induced excitation. Under

the conditions imposed in Series II some effect upon variation of performance might well be expected. Yet when  $P.E._\theta/\theta$  is plotted as a function of  $\theta$  (Fig. 4) it is found that there is no divergence whatever

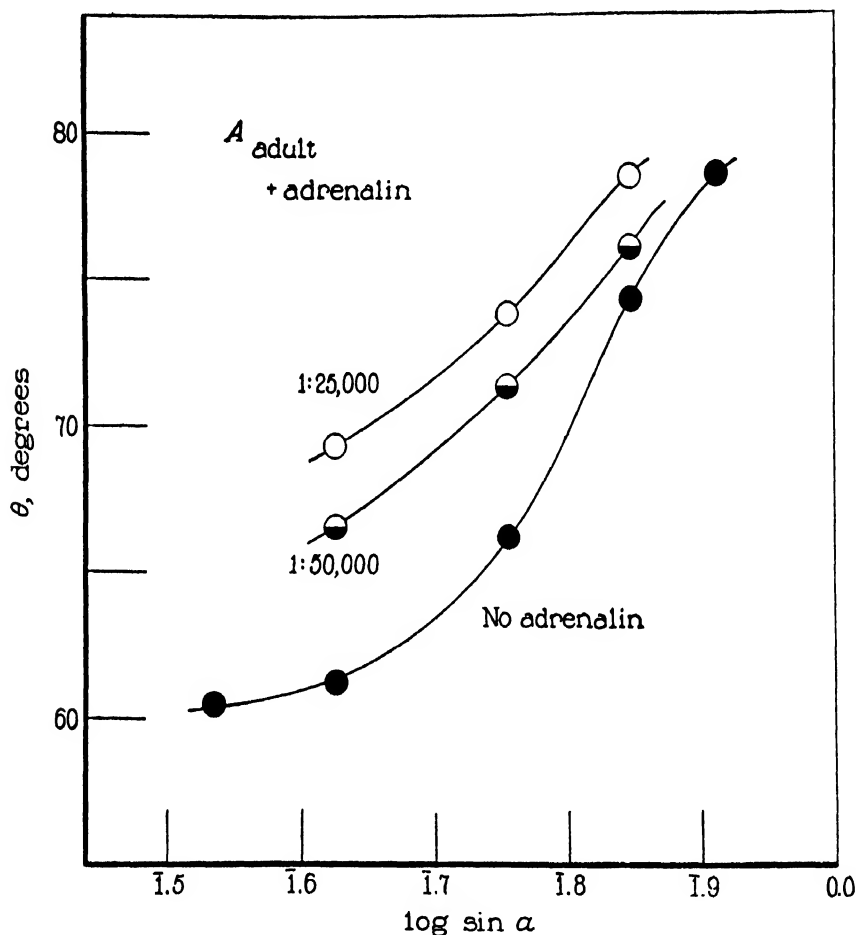


FIG. 3. Mean orientation angles ( $\theta$ ) for adult rats, race A, Series II;  $N = 4$  (3 ♂♂, 1 ♀),  $n = 45$ ; data in Table III. Solid circlets, no adrenalin; half circlets, adrenalin injection, 1:50,000; open circlets, 1:25,000.

from the relationship established for the uninjected adults in Series I or in Series II.

#### V

The  $\theta$  vs.  $\alpha$  curves for the rats in Series I and II differ significantly in the magnitudes of mean  $\theta$ . This is a matter of the ages of the rats in

the two sets. Nonetheless, the indices of variation are identical; for adults of other strains, as we make clear elsewhere, the indices are quite different. The rats in Series I were  $5\frac{1}{2}$  months old, those in Series II, 4 months old. The difference between the two curves is not one due to weights of individuals; it is of interest to notice that whereas the weights of the males (124 to 128 gm.) are in excess of

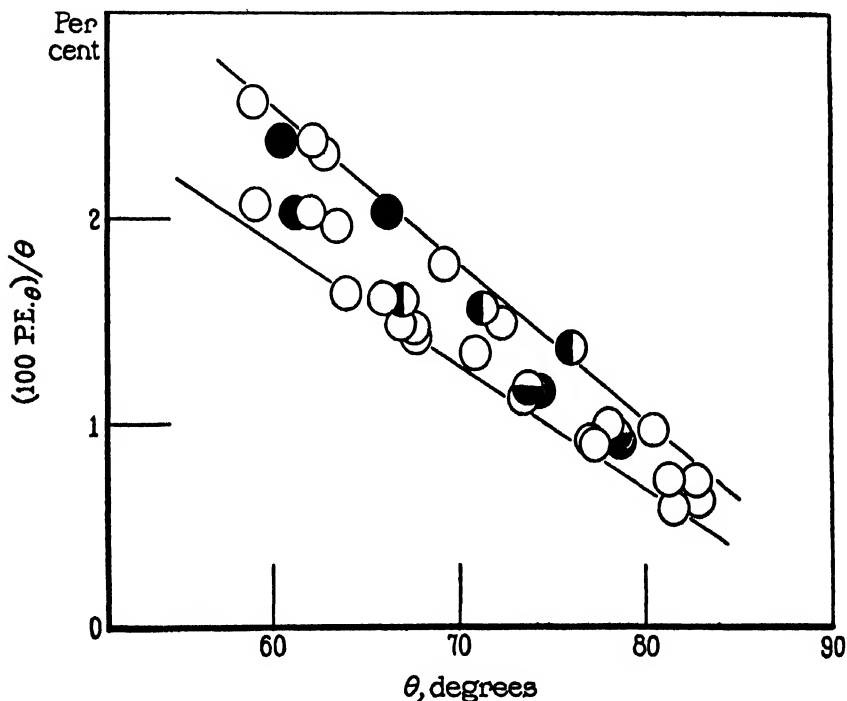


FIG. 4. Relative variations of orientation angles, adult *A* rats of Series II (*cf.* Table III and Fig. 3), are identical with the values found for Series I (Fig. 2). Open circlets, Series I; solid circlets, Series II without adrenin; ◐, adrenin 1:50,000; ◑, adrenin 1:25,000.

those of the females (106 to 109 gm.), the orientation angles for males and for females are identical. This agrees precisely with the findings when young rats are employed. As in the cases of the experiments with added weights (*cf.* Crozier and Pincus, 1931-32*b*), the findings summarized in Fig. 4 would be physically impossible if the observers introduced errors of recording; the variability with which we deal is an organic property of the rats.

From this standpoint it is evident that the proportion of controllable variance of orientation is an organic invariant. We have already indicated that it may be understood as being determined primarily by the number of receptor units implicated (Crozier and Pincus, 1931-32 *c*; *cf.* also Wolf and Crozier, 1932-33). The argument here derives in part from the independently justified use of  $\Delta(P.E._\theta)/\Delta\theta$  as a measure of the dependence of the variation of  $\theta$  upon the intensity of the excitation which  $\theta$  measures (Crozier and Pincus, 1926-27); and in part from the way in which the total variation is found to depend upon the relative number of receptor units available (Crozier and Pincus, 1929-30 *a*). The observed resistance of the proportionate modifiable variation to change, and its specific association with constancy of genetic types of rats, are of course entirely consistent with this view—and scarcely with any other.

This must not be supposed to imply that variability of orientation, taken as a test case for the study of “variability of behavior,” will not be found open to modification, and indeed in various ways. We have already pointed out (Crozier and Pincus, 1931-32 *d*) that diverse answers will be obtained, in a given case, according to whether one or another arbitrary criterion of variation is selected whereby to measure. For example, if observations of orientation were to be restricted to one slope of surface, we should have to say (1) that the performance of the adults is less variable than that of the young, (2) that the effect of adrenin is to decrease variation of  $\theta$ , in the adults, but (3) to increase it in the young; and no one of these statements would be in any useful sense correct. The outcome of the present experiments, we are satisfied, is to show that methods may be developed for such studies which will make it possible to see precisely what we mean when we seek to ask if a given experimental procedure or set of conditions has any real effect whatever upon variability of the performance of an organic system. Such methods depend upon the measurement of variation as a function of significant variables, and they are important for a diversity of reasons (*cf.* Crozier, 1929).

#### SUMMARY

The upward geotropic orientation (angle  $\theta$ ) of adult rats (race *A*) has been measured as a function of slope of substratum. The relative

variation of orientation angle is a declining rectilinear function of  $\theta$ . The fraction of the total observable variation of performance ( $\theta$ ) which is controlled by the intensity of excitation (56 per cent) is identical with that found for young rats of the same strain, although the total variation is a little greater.

Injection of adrenin distorts the  $\theta$  vs.  $\alpha$  graph in a manner quite concordant with the effect obtained in young rats. With the adults the absolute magnitudes of the variations of  $\theta$ , at corresponding intensities of excitation, are not affected by the action of adrenin, and, as with the young, the proportion of modifiable variation of  $\theta$  is not altered. The variability of performance, considered as a function of the performance, must therefore be regarded as an organic invariant. Certain consequences of this finding are referred to.

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# STIMULATION BY MINERAL AND FATTY ACIDS IN THE BARNACLE *BALANUS BALANOIDES*\*

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A previous study on the stimulating efficiency of the salts of the first seven normal aliphatic acids at constant pH ( $= 8.1 \pm 0.15$ ), on the barnacle *Balanus balanoides*, indicated increasing effectiveness as the length of the carbon chain increased (Cole, 1931-32). The data were presented graphically by plotting per cent closure, or area under plots of percentage difference in number open at the end of 8 minutes, against molar concentration. As a first approximation smooth curves were drawn through the points, and the assumption was made that a normal distribution of thresholds existed in the population of animals used. It was pointed out that formate seemed to be out of place in the order of effectiveness if 50 per cent closure or less (instead of 60 per cent or more) were taken as the criterion of response.

A complementary study of stimulation by the fatty acids has been made by allowing the pH to vary according to the amount of acid added to sea water. In addition, three mineral acids have been tested. Any effect due to the alteration in the ( $H^+$ ) should be identified by this method. Such an effect has been demonstrated, and a more detailed analysis of the response as related to the ( $H^+$ ) and to the length of the carbon chain has been made possible.

The experimental procedure used was similar to that previously described, except in the making of solutions. The following acids at from 8 to 14 concentrations each were used: hydrochloric, 0.0002 to 0.0025 N; sulfuric, 0.0004 to 0.0025 N;

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\* The experiments were done at the Mt. Desert Island Biological Laboratory during the summer of 1932.

† A part of the expenses of this investigation was met by a grant from the National Research Council for 1932.

nitric, 0.0002 to 0.00253 N; formic, 0.0002 to 0.002 N; acetic, 0.0002 to 0.003 N; propionic, 0.0004 to 0.002 N; butyric, 0.0002 to 0.002 N; valeric, 0.0004 to 0.002 N; caproic, 0.0002 to 0.002 N; and heptylic, 0.0002 to 0.0014 N. The ( $H^+$ ) varied from  $0.32 \times 10^{-7}$  to  $58.89 \times 10^{-7}$ .

18 liters of each solution were made by adding the desired amount of acid to fresh sea water. The mixture was thoroughly shaken and left standing until the ( $H^+$ ) as measured by the quinhydrone electrode became relatively constant. This interval was  $45 \pm 15$  minutes. Since sea water is buffered chiefly by bicarbonates, the addition of an acid to the system liberates carbonic acid and the change in pH is an approximate measure of the amount of carbonic acid produced when all of the acid added has reacted with the bicarbonates. Under these conditions the pH of sea water will change by about the same amount when equivalent concentrations of strong and weak acids are added. If the same concentrations, however, are made up in carbonate-free sea water adjusted to a pH of 8.3, the pH of the solution will change in accordance with the strengths of the acids added. Furthermore, if more acid is added than can react with the bicarbonates, then the pH of untreated sea water similarly changes in accordance with the strength of the acid used.

Each solution was divided into three parts and each part was tested by a different observer<sup>1</sup> on a different group of barnacles, totaling 222 individual barnacles. The rate of flow of solutions over the animals was  $250 \pm 15$  cc. per minute, and the temperature was  $17.0 \pm 0.3^\circ C$ . The number of regularly active animals (Cole, 1932) was recorded at 1 minute intervals and plotted as ordinates against time in minutes. The three plots for each solution were so nearly alike that averaging of the ordinates was justified. From the combined data the percentage of animals closing during each minute interval was calculated as a percentage on the basis of the number of animals open at the end of the interval as compared to the number open at the beginning of the test.

Two methods of treating the data were used. The first was similar to that employed for the fatty acid salts (Cole, 1931-32). Per cent closure was plotted against ( $H^+$ ) and smooth curves were drawn through the points. A family of exponential curves rising sharply from the origin, becoming asymptotic at progressively greater ( $H^+$ ), and revealing a graded series of constants, was displayed. The relationships showed that in the lower range all the acids were about equally effective as stimulating agents, but that as the ( $H^+$ ) increased effectiveness increased with the number of  $CH_2$  groups. Very little difference between the mineral acids and the first three fatty acids was

<sup>1</sup> The writers are indebted to Miss Helen H. Smith and Mr. Irwin W. Sizer for assistance in making observations and in many other ways.

apparent, but beginning with butyric acid the spread of the curves decreased and steadily became less through heptylic. The stimulating effect of the fatty acids is clearly related to the disturbances in the environment which are produced both by the non-polar portion of the molecule and by the change in  $(H^+)$ . Compared with fatty acid salts at constant pH, the free acids are about ten times more efficient as stimulating agents for the barnacle.

For preliminary interpretation the following development is helpful. If the amount of change in the number of animals open ( $c$ ) as compared with that of the intensity of stimulation ( $x$ ) is proportional to ( $c$ ) then the following equation may be written

$$\frac{dc}{dx} = -kc. \quad (1)$$

Upon integration, equation (1) becomes:

$$\frac{c}{c_0} = e^{-kx}, \quad (2)$$

where  $c_0$  is the original number of animals open. To show the relationship between per cent closure and intensity of stimulation, equation (2) may be revised as follows:

$$\text{Per cent closure} = 100 - 110e^{-kx}. \quad (3)$$

The total disturbance to which the animals respond within a fixed time is proportional to two forces, one related to the change in the  $(H^+)$  and the other to the field of force around the anion of the acid added to the environment. Both forces may be measured in terms of  $(H^+)$ , and the following expression may be developed from (3) to fit the data obtained at the end of a 4 minute interval:

$$\text{Per cent closure} = 100 - 100e^{-0.1z + (0.003125)2^n(z-0.4)} \quad (4)$$

where  $z$  is  $(H^+) \times 10^7$  and  $n$  is the number of carbon atoms (if present) in the anion of the acid.

A family of exponential curves, derived from equation (4), is reproduced in Fig. 1. The lowest curve, where  $n = 0$ , approximately describes the relationship between per cent closure at the end of 4 minutes and the change in  $(H^+)$  produced by adding one of the mineral

acids. The curve for formic acid, where  $n = 1$  (not shown in Fig. 1) is almost coincident with the one for mineral acids, lying only slightly above the latter.<sup>2</sup> No great difference between the stimulating efficiencies of the mineral acids and of formic acid appears, which means that equivalent concentrations of the anions enter into the equilibrium

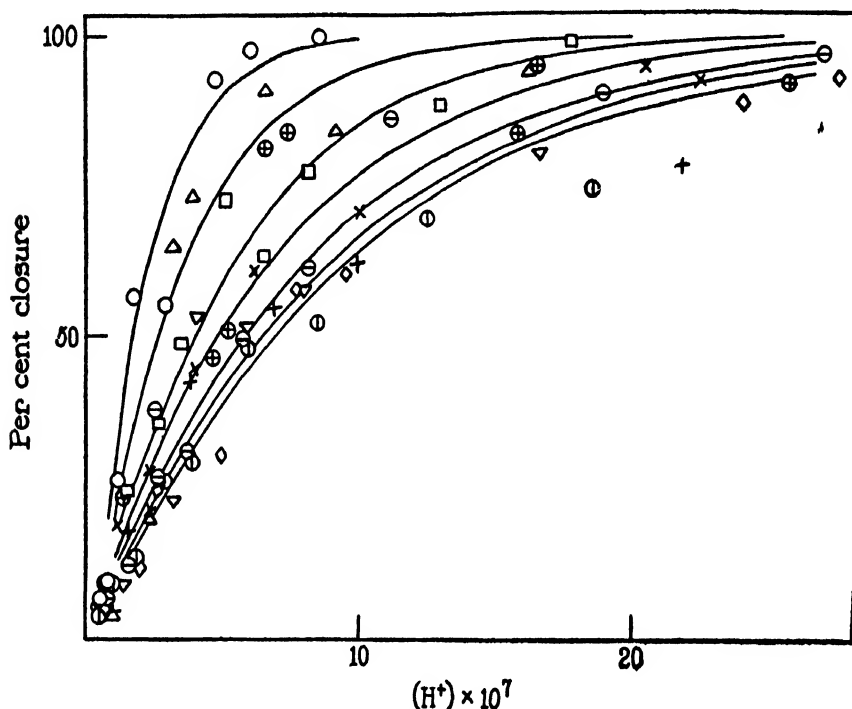


FIG. 1. A series of exponential curves derived from equation (4) in the text. The plotted points are average values obtained by using the following acids: HCl ( $\oplus$ ),  $\text{H}_2\text{SO}_4$  ( $\nabla$ ),  $\text{HNO}_3$  (+), formic ( $\odot$ ), acetic ( $\times$ ), propionic ( $\diamond$ ), butyric ( $\ominus$ ), valeric ( $\square$ ), caproic ( $\triangle$ ), and heptylic ( $\circ$ ) as stimulating agents on the barnacle. Temperature =  $17.0 \pm 0.3^\circ\text{C}$ .

of the system stoichiometrically. Structure of the anion appears to play no rôle in the case of these acids.

The next six curves, for acetic, propionic, butyric, valeric, caproic, and heptylic acids, represent the effect of increasing  $n$  from 2 to 7 inclusive. By adding these acids to sea water there is produced an

<sup>2</sup> The ordinates for the formic acid curve are on the average less than 1 per cent (of closure) higher than those for the mineral acid curve.

additional effect correlated with the structure of the anion and quantitatively determined by the number of  $\text{CH}_2$  groups. When the carbon chain contains 5 atoms or more this additional effect becomes approximately equal to the hydrogen ion effect. As would be expected, the difference between the members of the series becomes more and more marked as the concentration increases.

Deviations of the data from the theoretical interpretation just presented are noticeable, and appear to be greater than the experimental errors in measurement. A more complete analysis demands that these deviations be considered as significant. The second method of treating the data was to plot per cent closure against  $\log (\text{H}^+)$ , giving equal weight to each point.\* Unmistakable evidence of a compound sigmoid relationship appeared for each acid, as illustrated in Fig. 2. Although indications of the sigmoid character of the curves were noticed when  $(\text{H}^+)$  was plotted as abscissae, they became marked in the  $\log (\text{H}^+)$  plots. The amount of excitation, as measured by per cent closure, can therefore be considered more accurately as a logarithmic function of the  $(\text{H}^+)$ . Now if a normal distribution of one kind of receptors within the population were involved, an S-shaped curve should more clearly describe the data than the shape of the curves shown in Fig. 1. If more than one group of receptors, or if more than one group of thresholds, exists in the population, then the plots should indicate it by the number of summated sigmoid curves. The importance of such an analysis has been emphasized by Hecht (1927-28) and by Crozier and Pincus (1929-30, 1931-32) for other cases. When the differential of the curves,  $\frac{\Delta \text{ per cent closure}}{\Delta \log [(\text{H}^+) \times 10^8]}$ , is related to  $\log (\text{H}^+) \times 10^8$ , a significant series of trimodal distribution figures appears (Fig. 3). It may be assumed that each figure is the resultant of three approximately symmetrical distribution curves, indicating in the population of animals (or in any one individual) the existence of three chemoreceptor groups differing in mean thresholds. Overlapping of the groups is apparent, and the per cent closure is determined additively as the  $(\text{H}^+)$  increases.

It is impossible to know at this time the exact number of groups,

\*  $\log (\text{H}^+)$  was used in order (1) to plot conveniently the highest  $(\text{H}^+)$ , and (2) to spread out the points near the lowest  $(\text{H}^+)$  values.

whether they are morphological or functional units, and whether they are groups of receptors or of individuals. If the multimodal sensitivity is due to forces other than those correlated directly with the

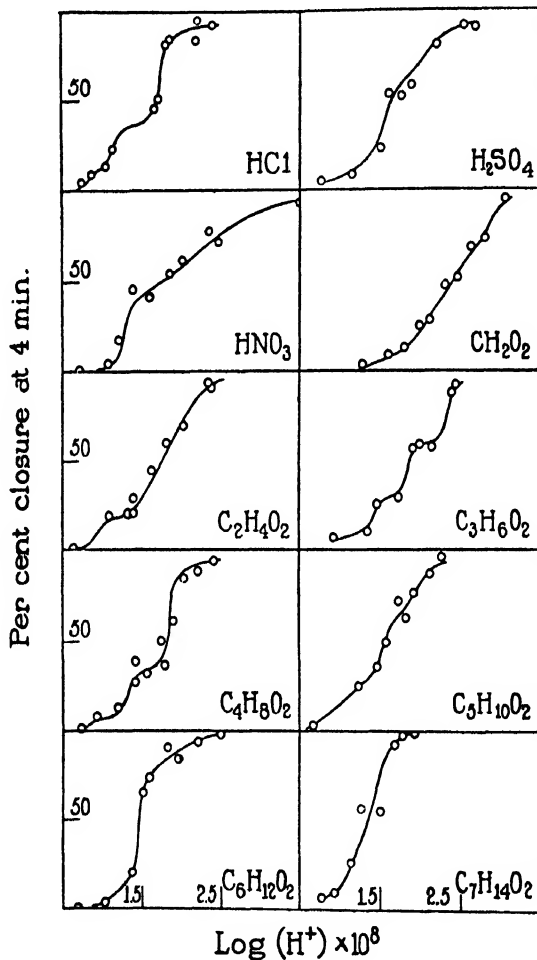


FIG. 2. Plots showing the compound sigmoid relationship between per cent closure and the  $\log (H^+) \times 10^8$  for hydrochloric, sulfuric, nitric, formic, acetic, propionic, butyric, valeric, caproic, and heptylic acids when used as stimulating agents on the barnacle. Temperature =  $17.0 \pm 0.3^\circ\text{C}$ .

stimulating agents then the system is a fluctuating one, and the phenomenon could not be correlated alone with the stimulating agent but also with other variables such as age factors and a fluctuating environment. Since exactly the same population of individuals was

employed for each acid, and since some checks were made on the reliability of former results over an interval of 10 weeks, it is believed that the three or more groups represent receptor groups with differen-

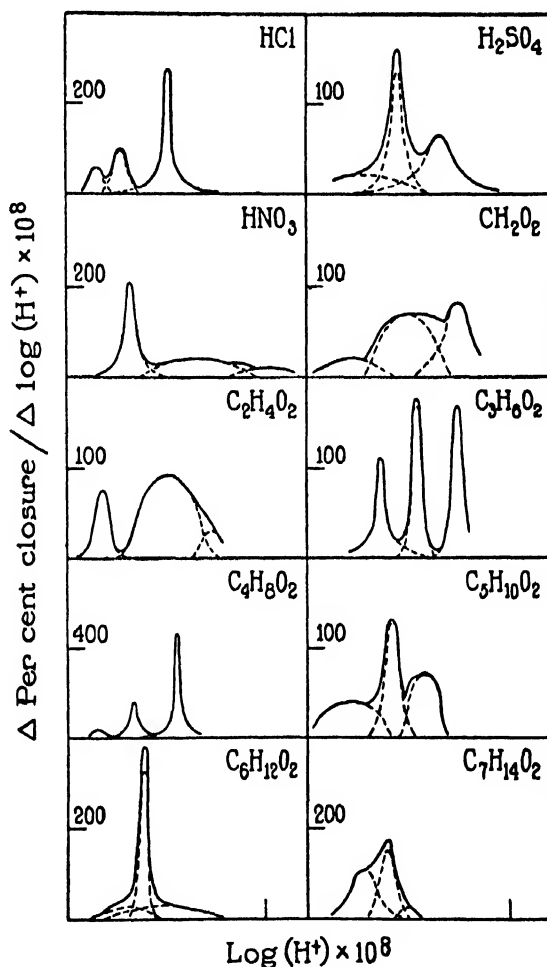


FIG. 3. Differential plots of the corresponding curves in Fig. 2. The scale along the  $x$  axis is identical to that in Fig. 2. Each figure is separated into three symmetrical distribution curves. For further explanation see text.

tial sensitivity. Information concerning the correlates of such differential sensitivity may be obtained from further investigation.

At any given  $(H^+)$  the stimulating forces attributable to the hydrogen ion are constant, and any difference in sensitivity of cor-



responding groups among the ten acids may be due to the difference in the field of force around the anion. The per cent closure obtained at a given ( $H^+$ ) is the result of the summation of the excitation of all three groups for each acid as presented in Fig. 2. If the degree of closure (from 60 to 80 per cent inclusive) is considered a measure of stimulation, equally effective hydrogen ion concentrations for the ten acids may be calculated and plotted against the type of anion (for example, the number of carbon atoms in the molecule), indicating the following order of stimulating efficiency for the fatty acids: heptylic > caproic > valeric > butyric = acetic > propionic = formic; and for the mineral acids: hydrochloric > sulfuric > nitric.

The second method of analyzing the data leads to essentially the same conclusion as the first one does; namely, that stimulation by mineral and fatty acids in the barnacle is correlated with two closely related factors, the ( $H^+$ ) or the potential of the cation, and the field of force around the anion. New information is revealed indicating the presence of three or more groups of receptors and their differential sensitivities, which explains why the first analysis is incomplete, why it does not show differences between the effects of the chloride, sulfate, and nitrate anions, and why the order of effectiveness among the fatty acids shows some variation from what might have been expected.

#### SUMMARY

1. Stimulation in the rock barnacle *Balanus balanoides* by hydrochloric, sulfuric, and nitric acids, and by the first seven members of the normal aliphatic acid series has been studied. The hydrogen ion concentrations of the solutions tested varied from  $3.2 \times 10^{-8}$  to  $5.889 \times 10^{-6}$ . The criterion of response was percentage closure in groups of individuals, recorded at 1 minute intervals until maximum closure occurred.

2. The intensity of stimulation by these acids is proportional to the effects of two forces, one related to the change in the ( $H^+$ ), and the other to the field of force around the anion of the acid added to the environment.

3. A preliminary interpretation of the results led to the develop-

ment of the following expression which fits approximately the data obtained at the end of 4 minutes:

$$\text{Per cent closure} = 100 - 100e^{-0.1s + (0.003125)2^n(s-0.4)}$$

where  $z$  is the  $(\text{H}^+) \times 10^7$  and  $n$  is the number of carbon atoms (if present) in the anion of the acid. This equation assumes that the anions of the mineral acids enter into the reaction stoichiometrically, and emphasizes the difference in the fields of force around the anion of the fatty acids, a difference which is correlated with the length of the carbon chain.

4. A further analysis of the data revealed the presence of three or more receptor groups which appeared to be differentially affected by forces originating from the anions of the acids.

5. The order of stimulating efficiency for the mineral acids was found to be:  $\text{HCl} > \text{H}_2\text{SO}_4 > \text{HNO}_3$ .

6. The order of stimulating efficiency for the fatty acids was found to be: heptylic > caproic > valeric > butyric = acetic > propionic = formic.

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# PHOTIC STIMULATION AND LEG MOVEMENTS IN THE CRAYFISH

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*(Accepted for publication, April 5, 1933)*

The doctrine of tropistic orientation of animals postulates that the stimulus acting asymmetrically upon the sense organs is mediated by the central and peripheral nervous system in such a way as to set up a state of differential muscle tonus on the two sides of the reacting animal. This results in the unequal action of the effectors, such as leg muscles, in such a way that the entire animal turns either towards or away from the source of stimulation. This is the doctrine as advanced by Loeb and since repeatedly found satisfactory as a probable physical basis on which to account for a multitude of observed phenomena. More important, it has permitted the formulation of equations which take into account the intensity and direction of action of the stimulus, the number and distribution of the sense organs affected, the position of the animal before, during, and after completion of orientation, and the kinds of leg and body movements. Such general formulae exist, for example, for geotropic orientation in some arthropods (Crozier and Stier, 1928-29; Kropp and Crozier, 1928-29), and in rodents (*cf.* Crozier and Pincus, 1932-33); and for phototropic orientation of various forms (Crozier, 1925-28; Mitchell and Crozier, 1927-28).

Orientation reactions resulting from unequal leg movements induced by changes at the receptors have been observed in the water scorpion *Ranatra* (*cf.* Crozier and Federighi, 1924-25). After removal of one eye the legs of each anatomical pair move in phase synchronously, in swimming, although the animal now makes circus movements towards the side of the intact eye. Such movements are unmistakably observed to result from the relative changes in amplitude of stride on the two sides,—decreased on the side of the intact eye and increased on the

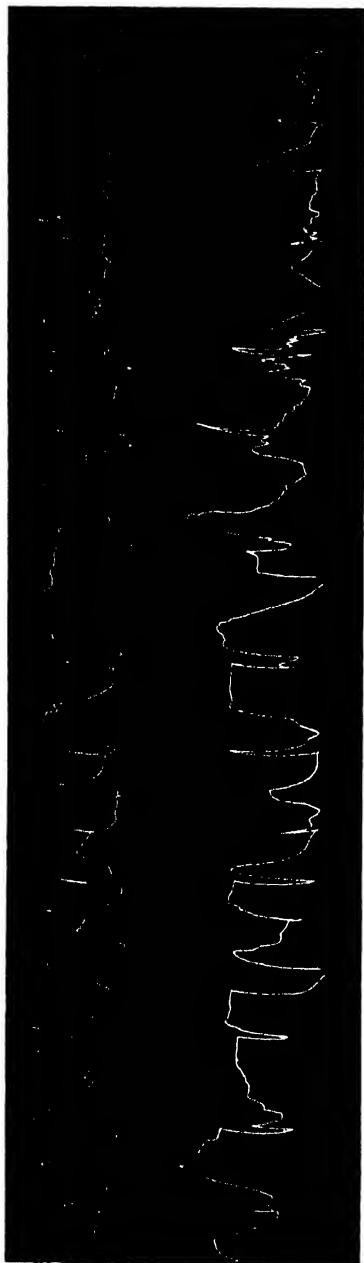


FIG. 1. In this and in the other figures the upper curve is a record of the movements of the second *right* leg of *Cambarus clarkii*, the lower curve shows that of the *left* leg. The source of illumination was directly in front of the eyes. Both eyes equally exposed to light; the legs on both sides move with the same frequency and amplitude.

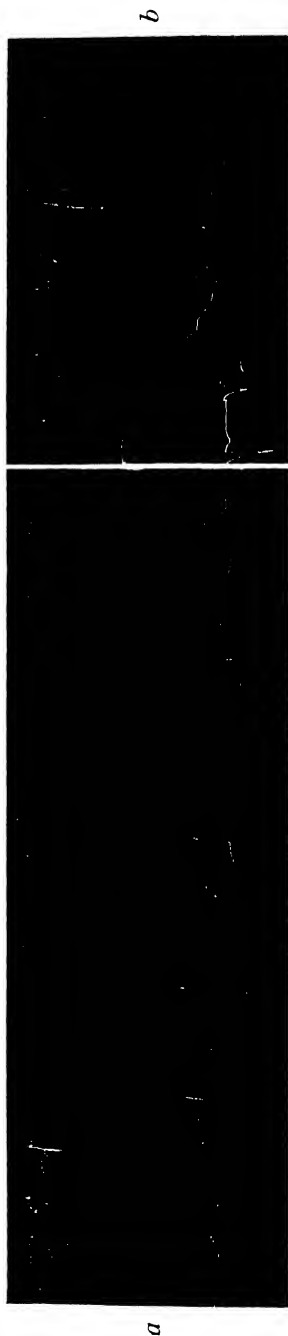


FIG. 2 *a* and *b*. The left eye of *Cambarus* was sealed with lamp black. The right leg moves with greater frequency and amplitude than the left leg.

opposite side. That such effects are due to the tonic effect of light stimulation on the leg muscles is also borne out by the determination of changes in amplitude of leg movements with changes in light intensity in intact arthropods (Welsh, 1932-33).

In the crayfish *Cambarus clarkii* it is possible to demonstrate a differential muscle tonus resulting from unequal illumination of the two eyes. The animal was fastened to a paraffin block and immersed in water, but the appendages were kept out of contact with the bottom and sides of the container. The second walking legs on right and left sides were connected at the distal end with a writing lever and the leg movements were thus recorded on a rotating kymograph drum. Obviously, the movements recorded included vertical sweeps of the legs and joint movements not ordinarily seen when the animal is walking. Such movements, however, were not very frequent and were equal on the two sides.

Fig. 1 shows the leg movements of an animal with both eyes equally exposed to light. There is no great difference either in frequency or in amplitude of movements. On covering one eye with lamp black and exposing the uncovered eye to light, a marked difference in leg movements on the two sides is apparent (Fig. 2). The movements of the leg on the side of the exposed eye are more frequent and are of greater amplitude. On covering the exposed eye, the leg movements again become approximately equal in frequency and in amplitude (Fig. 3). Due to certain practical difficulties, it was not possible to sever the nerve supplying the second walking leg; but a check was obtained by severing the nerve at the base of one eye-stalk. On exposing both eyes to light immediately after the operation, the leg on the side with the severed optic nerve shows greater activity than the normal, due probably to the effects of severing the nerve. Shortly afterwards the denervated eye is dropped off, and thereafter the leg on that side acts as though it were on the side of a normal but covered eye (Fig. 4). With both eyes functional, leg movements of the two sides are equal in number but do not occur synchronously. With both eyes covered, however, movements were both synchronous and equal. According to these results the animal should be photonegative, which in fact it is.



FIG. 3*a*. Both eyes covered with lamp black. The legs move synchronously with the same frequency and amplitude



FIG. 3*b*. Same as 3*a*, but on a slower drum, showing bursts of activity separated by periods of tonic immobility

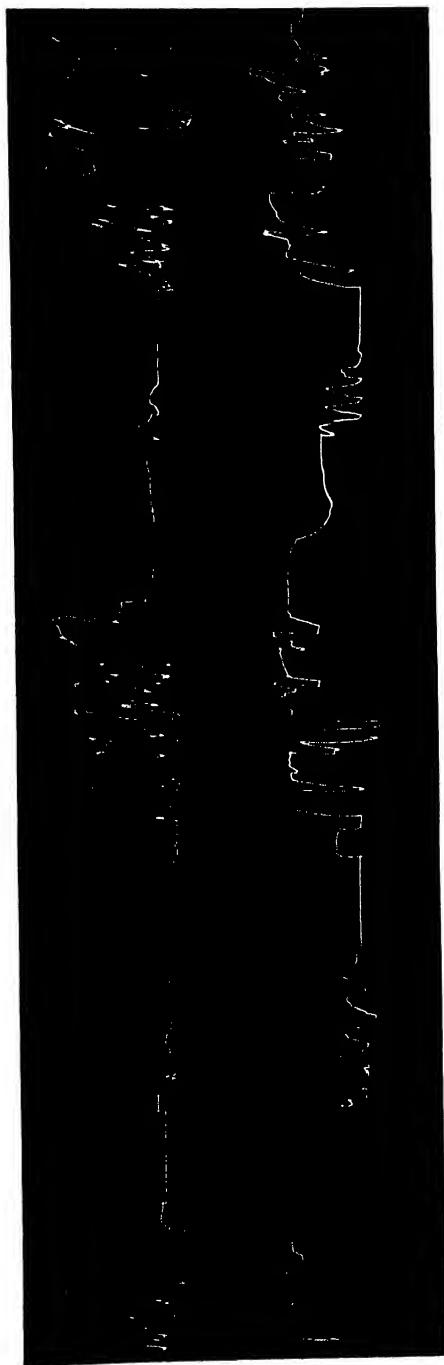


FIG. 4. Leg movements of a crayfish immediately after the right optic nerve has been severed. The leg on the blinded side moves more frequently and with greater amplitude.



## SUMMARY

When *Cambarus clarkii* is exposed to a source of light so that both eyes are equally illuminated, leg movements of the two sides are equal in frequency and amplitude.

On covering one eye and exposing the uncovered eye to light, leg movements on the side of the uncovered eye are more frequent and are of greater amplitude than on the side of the covered eye. On covering the exposed eye also the leg movements on the two sides again tend to become equal in frequency and amplitude.

When one eye is lost and the other remains functional, the leg movements on the side of the lost eye will be similar to those on the side of a normal, covered eye.

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# QUANTITATIVE ASPECTS OF CUTANEOUS SENSORY ADAPTATION. I\*

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## I

A method has been described for recording action potentials in single nerve fibres from single tactile receptors in the skin of the frog (Adrian, Cattell, and Hoagland, 1931). Leads were placed on one of the dorsal cutaneous nerves cut near the skin to record the antidromic impulses which are produced in a suitably selected single fibre by stimulation of some cutaneous area (*cf.* Hoagland, 1932). The action potentials were amplified and then recorded with a loud speaker and with a Matthews oscillograph used with a rotating mirror and camera. Stimulation of any desired frequency, duration, or intensity was secured by applying to the skin surface a jet of compressed air interrupted by a toothed disc fitted to a dental drill and revolved by a motor.

The receptors of the frog's skin were found to adapt very quickly to a continuous air jet, the rate of adaptation being comparable to that of an ordinary nerve fibre and thus differing from the slow adaptation of stretch receptors in muscle (Matthews, 1931).

With repeated air blasts of short duration, *i.e.* about  $5\sigma$ , only a single impulse is set up for each puff of air. If the frequency of stimulation is sufficiently great the end organ soon fails to follow every stimulus, more and more impulses being dropped out until the response ceases entirely. In a previous paper Cattell and Hoagland (1931) examined this failure, which they regarded as an example of sensory adaptation; the rate of failure depends primarily upon the frequency of stimulation, the duration of the stimulus, and the inter-

\* The experiments described in this paper were carried out in the Physiological Laboratory at Cambridge University, thanks to the kindness of Dr. E. D. Adrian who made available to me the necessary facilities.

val between the stimuli. The time for complete adaptation, up to the complete failure of responses, was found to vary from a few seconds at high frequencies of excitation to more than an hour at low frequencies.

## II

For the purposes of these experiments "adaptation" may be defined by measuring its rate as the speed of decline of frequency of the electrical responses in a single afferent fibre when an intermittent air pulse of constant frequency from a constant-pressure source is applied to the skin, at a fixed distance from it. Preliminary experiments with different discs showed that after the beginning of failure of response to continuing intermittent stimulus, the curve describing the failure

TABLE I  
*Duration of Stimuli for All Discs =  $5.6\sigma$*

No. of notches in disc	$\frac{\text{Stimuli}}{\text{Second}}$	$\frac{\text{Stimulus time}}{\text{Resting time}} = S/R = \frac{\text{Area removed}}{\text{Area retained}}$
1	17.5	0.111
2	35.0	0.251
3	52.5	0.428
4	70.0	0.670
5	87.5	0.994
6	105.0	1.500

of response in any given experiment follows either one or another of two simple empirical formulas. To ascertain the possible significance of these formulas, adaptation was measured as functions of various time relations of the stimulus as determined by properties of a series of discs. In this way, for example, the rate of decline in frequency might be measured as a function of the size of the notches in the disc, and as a function of the duration of the rest period between stimuli, as determined by the amount of solid area left between the notches.

A series of six celluloid discs of 26 mm. diameter was constructed, with from one to six notches in each disc. The notches were carefully cut so that they were all of equal size, corresponding to a constant duration of stimulus of  $5.6\sigma$  when the disc was rotated at 17.5 revolutions per second, the speed used throughout the experiments. Certain significant properties of the series of discs are given in Table I.

To facilitate changing the discs during an experiment, a method devised by Dr. McKen Cattell and used by us in our previous experiments was employed. One of the notches of each disc was continued through to the centre, so that the disc could be slipped on and off between small metal plates fitted to the end of the dental drill shaft and forced together by a spring. In this way, not only was changing the discs facilitated but the position of the air nozzle clamped to the drill immediately above the revolving notches was kept fixed—a matter of considerable importance in maintaining constancy of stimulation throughout an experiment.

Fig. 1 is a reproduction of the series of discs. In all of the preparations used brain and cord were pithed so that no movements of the frogs occurred after setting the nozzle in position above the receptor.

Fig. 2 gives typical photographic records for two preparations showing the decline in frequency of impulses after failure begins. The disc used was No. 5 (Table I), which causes rapid adaptation.

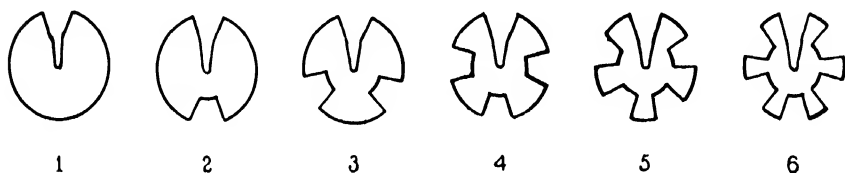


FIG. 1. Outlines of series of discs  $\times \frac{1}{2}$ .

The method of averaging employed may possibly have a bearing on the form of the equations here used to relate frequency of response to time. The procedure consisted in counting the number of impulses in a convenient period as measured by the time marked on the record after adaptation had begun to manifest itself by the first dropping out of an impulse in the series of responses to the stimulus. Since the frequency of stimulation varied from disc to disc, and since the number of impulses per unit time on the records decreases rapidly with adaptation, it was convenient to use a kind of moving average in which the times corresponding to a convenient number of impulses were measured. Trial computations showed no discrepancies in the forms of the curves of adaptation with wide variations in the number of impulses in the units used for averaging.

Fig. 3 is an example of the typical decline in frequency (expressed as percentage of the maximal frequency), plotted against the time from the first appearance of failure to respond, for Discs 1, 3, and 6. The points represent two sets of random observations for each of

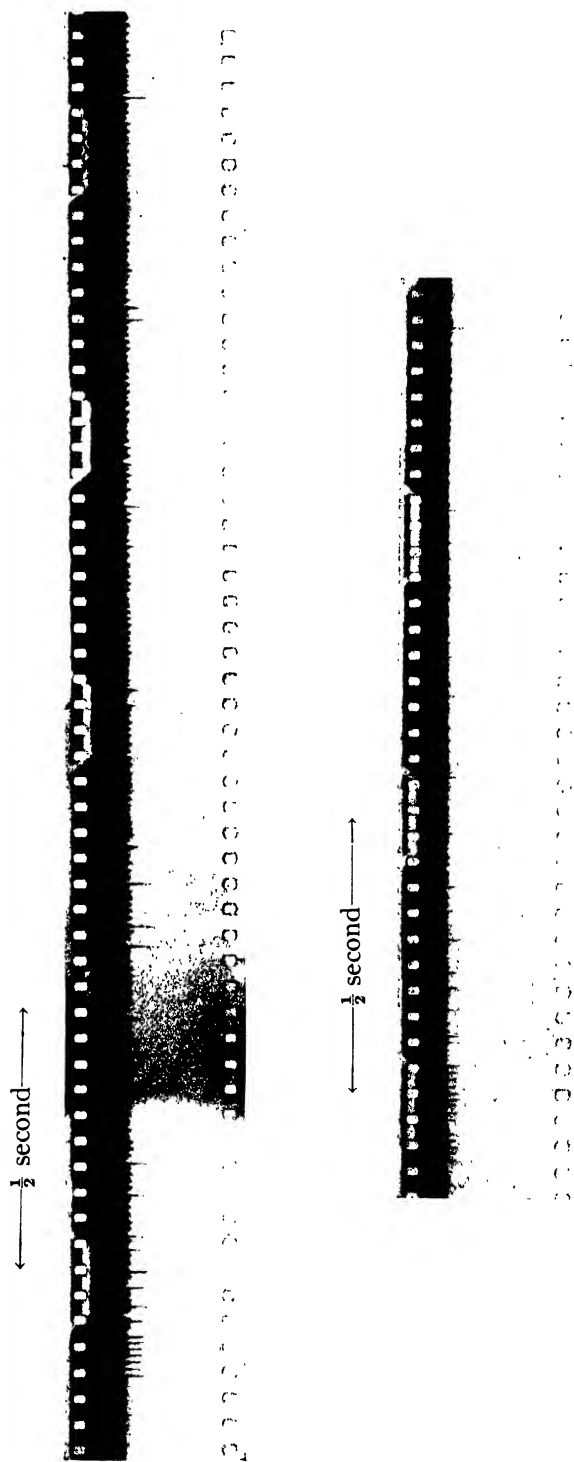


FIG. 2. Typical records showing, in two preparations, the decline in frequency of impulses after beginning of response.

these discs taken over a period of about half an hour. The good fit of the curves to both these sets of observations shows that no irreversible changes influencing adaptation had taken place in the course of the experiment.

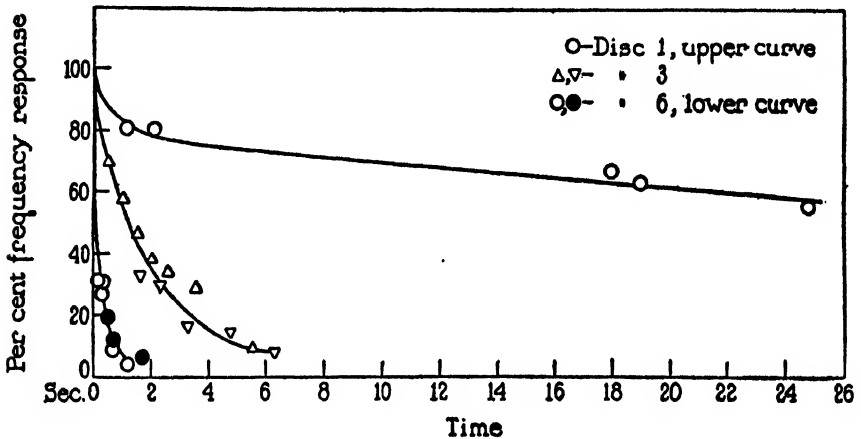


FIG. 3. Plot of per cent frequency of response against time, after the first failure of response to intermittent stimulation for Discs 1, 3, and 6. The experiment shows two sets of observations for each disc.

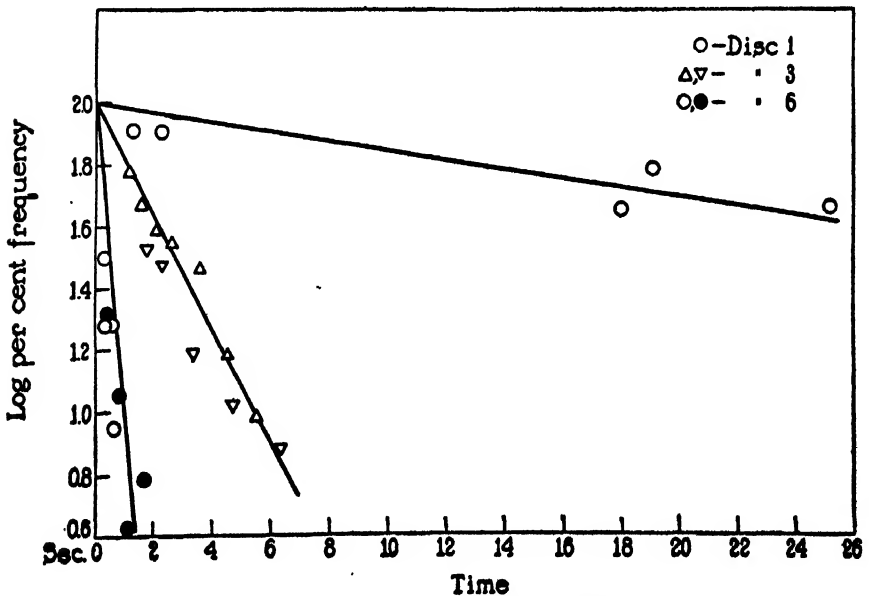


FIG. 4. Rectified form of the curves of Fig. 3.

Fig. 4 gives the rectified form of these curves; it is seen that they are fairly described by the equation

$$t = -k \log f + C,$$

where  $t$  is the elapsed time,  $f$  is the corresponding per cent frequency of response, and  $-k$  is the measure of adaptation, the velocity constant of the process of adaptation.

This empirical equation is essentially of the form of that of a first order process, if it should be assumed that the frequency depends upon the concentration of a substance which is used up during stimulation. Adaptation is also seen to be more rapid to discs with larger numbers of notches and with relatively higher ratios of  $\frac{\text{Stimulating time}}{\text{Resting time}}$  more conveniently written  $S/R$ . This obvious increase in rate of adaptation ( $-k$ ) with increase of  $S/R$  is suggestive when one considers the apparently reversible nature of the adaptation process. We may picture adaptation as occurring during the time that each stimulus is on and recovery taking place reversibly at the same time but with the recovery greatly accelerated during the intervening rest periods, the value of  $-k$  being essentially proportional to the equilibrium constant for the reversible system.

To test the effect of stimulus duration and of the rest period duration of the revolving discs on  $-k$ , a series of twenty-eight preparations was examined. The skin was stimulated by the interrupted air jet and the responses were photographed. For discs with few notches and with relatively long rest periods, such as Disc 1, the impulses were found to follow the stimulus for several minutes with a subsequent progressive decline in frequency which might last an equally long period. To conserve photographic paper, sample records were taken at measured intervals of from 5 to 20 seconds. With the discs above Disc 2 in the series it was usually possible to photograph the complete adaptation on one record as is shown in Fig. 2 with Disc 5. The first disc tested was generally No. 6, which produced the maximal adaptation of the series, and this first disc was then used as a control. After obtaining records in haphazard order from all the discs of the series, the first disc, generally Disc 6, was again used. If the rate of adaptation to this control disc as recorded by the loud speaker, and subsequently verified quantitatively from the photographs, was the same at the end as at the beginning of the experiment, lasting about 20 minutes, the preparation was assumed to have suffered no irreversible changes. This test of the integrity of the preparation is important since many preparations show progressive failure despite care in shielding the nerve

from the air and in bathing the tissues with fresh Ringer's solution. Out of twenty-eight preparations, seventeen only showed constancy of adaptation to the control disc. The remaining eleven experiments were accordingly discarded as irrelevant to the present discussion.

Room temperatures were recorded by a thermometer placed near the preparation and fluctuations of more than  $0.2^{\circ}\text{C}$ . were seldom recorded during an experiment. There was little day-to-day fluctuation of the room temperature, which remained at approximately  $17 \pm 1^{\circ}\text{C}$ . The stimulating air used was essentially at the temperature of the room, since it had ample time to cool after compression in a large storage tank and was piped for a considerable distance through the building. The jet blown on a thermometer bulb did not change its temperature by as much as  $0.1^{\circ}\text{C}$ . Cooling of the skin by evaporation was prevented by painting it with oil. The nerve was shielded from the stimulus by drawing it into a glass tube containing the leads to the electrical recording system and packing cotton soaked in Ringer solution around both ends of the tube. Moist packing was also placed around the incision, including the millimeter or so of nerve between the junction with the animal and the entrance of the nerve into the glass tube.

To compare the effects of the ratio  $S/R$  for different discs the per cent decline in frequency was calculated; 100 per cent was the maximal frequency of response, corresponding to a one-to-one relation with the stimulus frequency of the disc. In this way absolute differences in adaptation rates due to the fact that the disc stimulates at different frequencies were eliminated and the relative adaptation rates became comparable.<sup>1</sup>

### III

Two different forms of adaptation curves were obtained with the seventeen preparations successfully examined. The first type of result has already been mentioned and is given in Figs. 3 and 4. Here adaptation is typically described by the equation.

$$t = -k \log f + C.$$

The second result is represented by characteristic adaptation curves which, while superficially resembling the curves of Fig. 3, differ fundamentally in the type of empirical relation describing them. Fig. 5 is the typical plot of such an experiment for three of the discs, and Fig. 6 shows these curves rectified. The logarithm of the time is nearly

<sup>1</sup> In the present experiment it has not been possible to vary  $S/R$  from disc to disc without also varying the frequency of stimulation (*cf.* Table I).



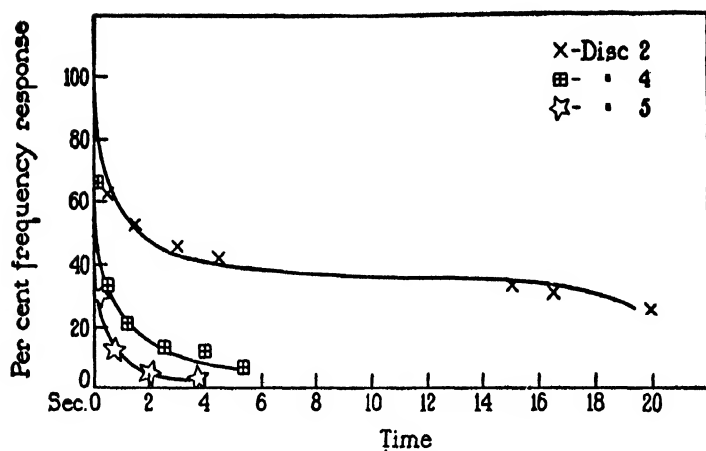


FIG. 5. Plot of per cent frequency of response as a function of time for three discs showing rapid early failure of response as contrasted to the curves of Fig. 3.

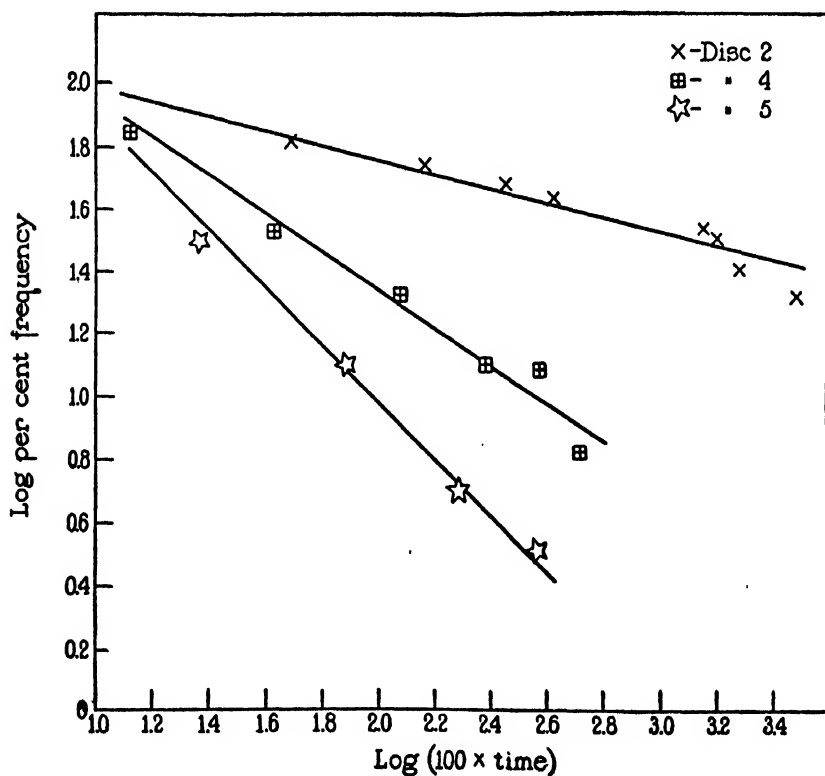


FIG. 6. Rectified form of the curves of Fig. 5.

enough an inverse linear function of the logarithm of the per cent frequency. The curve is hyperbolic, described by the equation

$$t = a f^{-b}$$

where  $f$  and  $t$  are respectively frequency and time, and  $a$  and  $-b$  are constants. The constant  $-b$  is defined as the adaptation rate in the sense in which  $-k$  was defined above. This comparison of  $-k$  and  $-b$  is obvious when logarithms are taken on both sides of the hyperbolic equation,

$$\begin{aligned}\log t &= \log a - b \log f \\ \log t &= K - b \log f\end{aligned}$$

$-b$  is seen to be the slope of the line relating  $\log t$  and  $\log f$ .

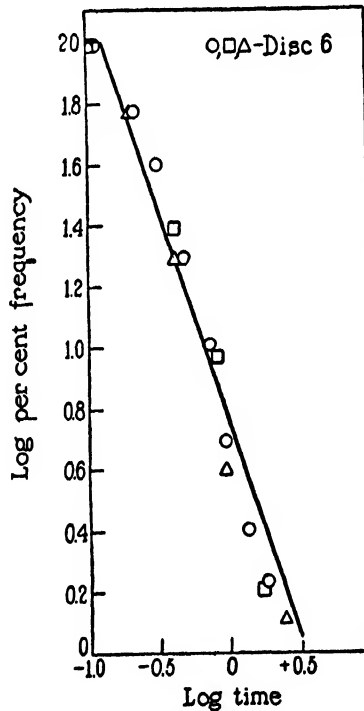


FIG. 7. Figure showing constancy of response to control Disc 6 over an interval of about 20 minutes. The plot shows three sets of data taken at intervals of from 5 to 10 minutes during an experiment.

Fig. 7 is a plot of *per cent frequency vs. time* from three records with Disc 6 made at intervals of from 5 to 10 minutes during an experiment. The plot shows great constancy of response to Disc 6.

## IV

The difference in the forms of the two equations for adaptation rate suggests possible uncontrollable experimental differences, and these were accordingly looked for but were not found. The preparations

TABLE II  
*Values of  $-k$  as Decimal Parts of the Values for Disc 6*

Disc No. ....	1	2	3	4	5	6
Experiment No.						
6	0.0504		0.38			1.00
8 A	0.0305			0.305		1.00
8 B		0.146		0.57		1.00
9	0.0380		0.258			1.00
11 A			0.00296	0.221		1.00
11 B	0.065			0.325		1.00
13 A		0.00348	0.348	0.665		1.00
13 B		0.0200	0.145	0.109	0.713	1.00
15 A	0.0094	0.088		0.161	0.456	1.00
Means . . .	0.0387	0.064	0.227	0.337	0.584	1.00

TABLE III  
*Values of  $-b$  as Decimal Parts of the Values for Disc 6*

Disc No. ....	1	2	3	4	5	6
Experiment No.						
4	0.0907		0.682	0.675	0.820	1.00
7	0.686	0.857	0.813	1.00	0.857	1.00
8 C				0.730		1.00
16 B	0.175			0.787	0.787	1.00
17 B		0.563	0.658	0.620	0.835	1.00
18 C	0.258	0.239		0.675	1.00	1.00
Means . . .	0.302	0.553	0.717	0.748	0.860	1.00

showing logarithmic adaptation were scattered quite at random among those showing hyperbolic adaptation. In several cases a particular nerve would show either the logarithmic or the hyperbolic adaptation while an adjacent nerve in the same frog tested imme-

diately afterwards would show the other of the two characteristic forms. In two cases a shift from the logarithmic to the hyperbolic form of curve occurred during the course of the experiment, despite the fact that adaptation to Disc 6 was maintained constant to the end. Of the seventeen preparations, nine were of the logarithmic type and six were of the hyperbolic type, while two showed a change from one type to the other in the course of the experiments. It so happens that in the figures presented logarithmic adaptation is shown as oc-

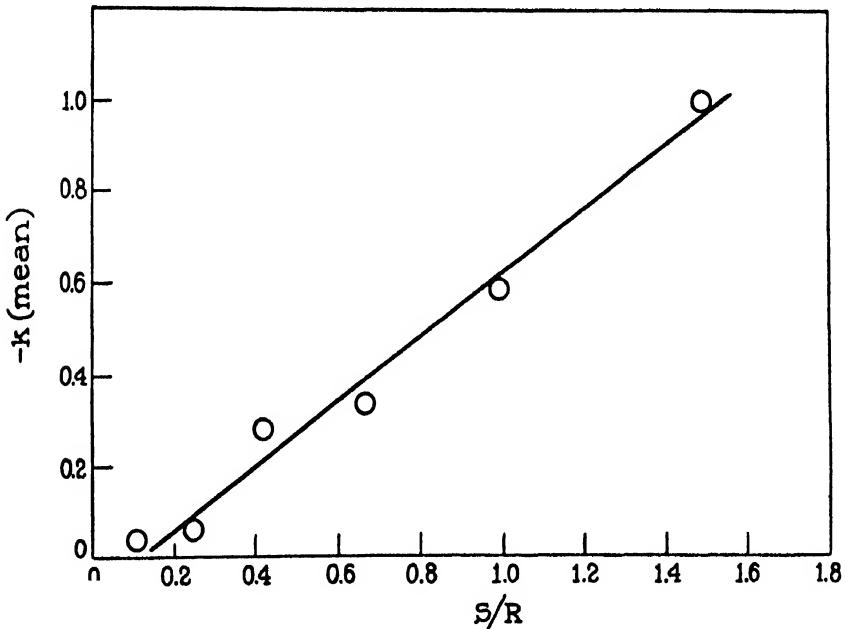


FIG. 8. Plot showing linear relation between the ratio  $S/R$  and the mean velocities of the logarithmic type of failure.

curing with Discs 1, 3, and 6, and hyperbolic with Discs 2, 4, and 5, but there is in fact no tendency for a particular disc to give a particular type of adaptation curve, as may be seen from Tables II and III. In numbering the experiments the numbers refer to individual frogs and the letters A, B, C, and D refer to different dorsal cutaneous nerve preparations in the same frog.

Fig. 8 is a plot of the mean values of the adaptation rate  $-k$ , as a function of the ratio  $\frac{\text{Stimulating time}}{\text{Resting time}}$  for each disc of the series.

Fig. 9 is a plot of the values of  $-b$  as a function of the logarithm of the ratio  $\frac{\text{Stimulus time}}{\text{Resting time}}$ .

While statistically the data are meager, especially more so for some discs than for others, a plot of the means is justifiable since plots for individual preparations, while showing considerable variation, follow, in general, the functions shown by the means.

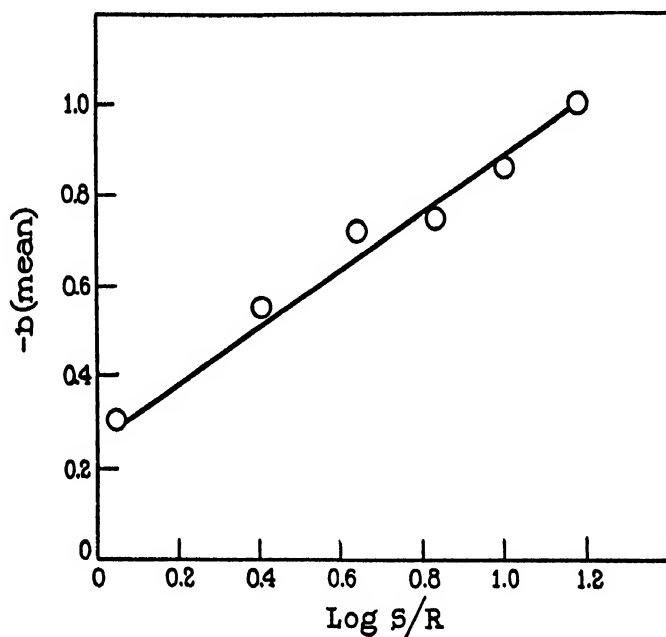


FIG. 9. Figure showing the linear relation between  $-b$ , the velocity of hyperbolic failure, and the logarithm of  $S/R$ .

v

The foregoing experiments were undertaken with a view to elucidating the kinetics of cutaneous sensory adaptation but the findings do not at present warrant much in the way of theoretical generalizations. It is clear that responsiveness of a receptor declines rapidly during the time that the stimulus presses on the skin and that this responsiveness returns partially in the brief interim between stimuli. Several paradigms have been proposed which could account for the quantitative aspects of this effect, but more experiments are necessary before it will be possible to decide in favor of any one mechanism.

The fact that some preparations give logarithmic adaptation while others give hyperbolic adaptation is difficult to account for. It is possible that these two forms may be limiting cases of a single general underlying equation. It is interesting in this connection to note that in two preparations clear shifts occurred from logarithmic to hyperbolic adaptation during the course of the experiments.

There are certain limitations to the method. One of these was the impossibility of keeping the frequency of stimulation and the duration of the stimulus constant when varying the ratio  $S/R$  from disc to disc. The ratio  $S/R$  may be shown to be equivalent to  $\frac{Fd}{1-Fd}$  where  $F$  is the frequency and  $d$  is the constant duration of the stimulus. It is clear, therefore, that frequency is a variable along with  $S/R$  and may modify the adaptation in some way independently of  $S/R$ . One can compare adaptations by using the per cent decline of the frequency of impulses as a measure of the response, but, experimentally, frequency of stimulation was a variable along with  $S/R$ .

#### SUMMARY

1. Adaptation of tactile receptors in the skin of the frog to excitation by an intermittent jet of air is measured and correlated with certain properties of a series of notched discs used to interrupt the air stream.

2. Adaptation in fifteen cases is found to be described by either one of two empirical formulas,

$$\begin{array}{ll} \text{or} & t = -k \log f + C, \text{ for nine preparations} \\ & t = a f^{-b}, \quad \text{for six preparations} \end{array}$$

where  $f$  is the per cent frequency at time  $t$  and  $-k$  and  $-b$  are constants defining the rate of adaptation.

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## REACTIONS OF VALONIA AND OF HALICYSTIS TO COLLOIDS

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It would be foolish to deny that protoplasmic surfaces are usually impermeable to colloids. And it is reasonable to assume that the size of the molecular aggregate plays an important part in this relationship. But it does not seem wise to close one's mind to the possibility that, under certain conditions, healthy protoplasmic membranes may allow the passage of specific colloids in amounts that are ordinarily not detectable by chemical methods. We must not forget that collodion capsules that will not permit the passage of colloids in quantities detectable by the best chemical procedure, can be proved to be semi-permeable by serological means. Thus agglutinogens, in tested collodion capsules, when placed in the body cavities of laboratory animals, produce agglutinins (See R. Paltauf).

Along one line of attack, the passage of proteins through the membrane or membranes separating the maternal from the fetal circulation, a considerable literature exists, which has been summarized recently by Ratner, Jackson, and Gruehl (1927). Various investigators have shown that the placenta is permeable to different proteins, such as antitoxins, precipitins, bacteriolysins, etc., in man, guinea pigs, and rabbits, though the placenta is not permeable to such substances in cows, goats, and sheep. Ratner, Jackson, and Gruehl interpret these conflicting results by pointing out that in man and in Rodentia a single cell membrane separates the circulations of mother and fetus, while in Ruminantia there are three cell layers.

Along another line of attack, there is the immense collection of facts connected with allergic manifestations on the skins of different animal species,—facts which can hardly be interpreted unless one



admits that proteins exert specific effects on the membranes of living animal cells.

Admittedly, such phenomena are hard to explain on the basis of our present knowledge, in the face of determinations of diffusion coefficients for proteins such as 0.059 for egg albumen and 0.014 for diphtheria toxin. But the chemistry of living matter is complex; and we are still comparatively ignorant of its fundamental theorems. We know little concerning the chemical rules at the point where solutions pass into colloidal systems, of the effects of mixed colloids, of the behavior of mixtures of electrolytes and non-electrolytes, of dispersing agents, of coagulating agents, of the limitations of the Donnan equilibrium, of previous activity (hysteresis), etc.

In this connection, some observations on *Valonia macrophysa* Kütz and *Halicystis Osterhoutii* Blinks and Blinks, made during February and March, 1932, at the Bermuda Biological Station for Research,<sup>1</sup> may be of interest. These two large-celled coenocytic algae, possessing protoplasmic membranes from  $5\mu$  to  $8\mu$  thick, are well known because of the extended use that Osterhout and his colleagues have made of them in physiological researches. The permeability of their protoplasmic membranes to crystalloids is probably more accurately known through this work than are the permeabilities of the cells of any other organisms.

These cells were collected in quantity, cleaned carefully, and kept in deep battery jars filled with sea water (pH by Hellige = 8.4), in diffuse light, — dead specimens being removed each day.

Cells used for experiment had been collected at least a week, and were apparently healthy unless otherwise noted. Whether they were actually healthy or not is unknown. Both species are easily disturbed by changed conditions, *Halicystis* being the more delicate. This disturbance can be measured accurately by changes in their electrical properties; but since the apparatus necessary for such measurements was not available to us, all we can say is that the normal death rate, under the laboratory conditions used, was about 1 per 1000 daily for

<sup>1</sup> The writers wish to express their gratitude to Director J. F. G. Wheeler and to Mr. W. Gleeson for many kindnesses, including much time spent in the collection of material; and to Dr. W. J. V. Osterhout and Dr. L. R. Blinks for advice on the care of the algae used.

*Valonia* and about 1 per 2000 daily for *Halicystis* after the first week subsequent to collection, provided dead cells were removed from the containers at least once a day.

The death of *Valonia* cells manifests itself in three ways. (a) In about 2 per cent of the cases, color is wholly lost in about 4 hours, without pseudoplasmolysis. The color due to chlorophyll simply disappears, the chloroplasts meanwhile retaining their shape. After the bleaching occurred, the chloroplasts were usually devoured by a protozoan ( $5\mu \times 2\mu$ ) which could not be stained for study in either gentian violet or hematoxylin. (b) In about 8 per cent of the cases, the protoplasm ceases to function properly, the chloroplasts slide together without bleaching immediately, and bare spaces appear where the cell wall is not underlaid with protoplasm. (c) The characteristic manner in which about 90 per cent of the *Valonia* cells deteriorate, however, is a sort of pseudoplasmolysis, in which the protoplasm gradually detaches itself from the cell wall, shrinking to about  $1/25$  of the bulk of the cell in approximately 12 hours. When this pseudoplasmolysis is complete, the cell wall has usually retained its rigidity; but it loses this characteristic within the next 6 hours.

*Halicystis* cells practically always deteriorate by the second method. They often became flaccid immediately after collection but regained turgidity within 6 hours if they lived.

There was a high death rate for several days after collecting in the case of the *Valonia* cells, due to handling and changed conditions, closely correlated with the size of the cell. After about 4 days, the death rate dropped sharply and remained almost constant.

1. *The Reactions of Valonia and of Halicystis to Peptone.*—There was apparently some penetration of peptone, as shown by the following experiment.

The peptone used was prepared at the Difco Laboratories (Lot 23368) and was obtained through the courtesy of Mr. H. G. Dunham. It contained 2.72 per cent of ash, 14.92 per cent of nitrogen in the form of peptone (*i.e.*, not precipitated by ammonium sulfate, though part may be amino acids), and the remaining nitrogen, 0.24 per cent, in the form of proteose. The product is of animal origin, but its exact source has not been divulged. Various lots of *Valonia* and of *Halicystis* were allowed to remain indefinitely in solutions of 1 per cent and of 2 per cent of this peptone, dissolved in sea water. The pH of each solution was 7.6, as tested by Hellige apparatus. The Posner biuret test was ++.

Neither *Valonia* nor *Halicystis* appeared to be noticeably affected by this environment. The death rates were only slightly higher than for controls during the 10 day observation periods used. Longer records were impossible because the peptone formed an excellent medium for the growth of a species of bacterium<sup>2</sup> which produced jelly-like cultures of such density that the water always became cloudy and gave off a rank odor by the end of the 10 day period. Such deleterious effects as were observed here were probably due to the bacteria rather than to the peptone, for a similar rise in the death rate was observed when the bacteria were planted in pure sea water, although the bacterial density was here much smaller. At all events, it was observed that both *Valonia* and *Halicystis* were able to withstand this treatment for 10 days, and were often able to live indefinitely when returned to sea water.

Twelve healthy *Halicystis* cells and a like number of *Valonia* cells, or small clusters of medium size, were placed in a 2 per cent solution of peptone in sea water and allowed to remain 6 hours. They were then thoroughly washed in sea water and dried carefully between filter papers. The *Halicystis* cells were apparently unaffected; the *Valonia* cells lost a little of their normal turgidity by this treatment. Six cells of each species were then tested for protein by Posner's modification of the biuret test. The test was made in this manner. The cells were placed in centrifuge tubes, punctured with gold needles, and centrifuged. The clear contents of the vacuoles were used. The results were: *Halicystis* ++, *Valonia* +, controls of each species —. The other six cells were replaced in sea water for viability observations. The *Valonia* cells gradually died, the last one succumbing during the fourth week. At the end of 3 weeks, all the *Halicystis* cells were still healthy.

This experiment was subsequently repeated, using a 1 per cent solution of peptone in sea water and an exposure of 2 hours. The biuret tests were negative for both species. The cells not used for the tests

<sup>2</sup> These bacteria frequently contaminated our dishes when ordinary laboratory finger-bowls were used as containers. No such difficulty was encountered with the use of battery jars. The bacterium thus appears to be highly aerobic. [Dr. Osterhout informs us that Dr. Blinks and he have observed growth of this bacterium in battery jars and in stoppered bottles.]

were returned to sea water and observed for 4 days. All remained alive at the end of that time, though they did not appear to be in perfect condition.

2. *The Reactions of Valonia and of Halicystis to Proteose.*—There was apparently some penetration of proteose, as shown by the following experiment.

The proteose used was the "bacto-protone" of the Difco Laboratories (Lot 23425), also received from Mr. Dunham. It consists almost entirely of proteoses of animal origin,—there being 12.23 per cent nitrogen in the form of proteose and 2.67 per cent nitrogen in the form of peptone. Various lots of *Halicystis* and of *Valonia* cells were exposed, indefinitely, to 1 per cent and to 2 per cent solutions of this proteose in sea water. The solutions showed a slight cloudiness, and were filtered before use. Each solution had a pH of 7.0, and was ++ to the Posner biuret test. As in the case of the peptone, both species exhibited a high degree of tolerance to the medium. The degree of tolerance to proteose appeared to be somewhat greater than to peptone. This effect may be due to the small amounts of amino acids that were probably present in the peptone and not in the proteose; or, it may be due to the fact that the proteose did not form quite so good a medium for bacterial development. The fact remains that the more resistant cells of both species will tolerate either 1 per cent or 2 per cent proteose in sea water for 10 days, and will live in an apparently healthy condition afterward when returned to sea water.

As in the earlier experiment, twelve healthy *Halicystis* cells and twelve cells, or small clumps, of *Valonia* were exposed for 6 hours to the action of 2 per cent proteose in sea water. They were then washed thoroughly and dried between filter papers. Half of each type were returned to sea water for viability observations, and the vacuole sap of the other half was tested for protein as before. The Posner biuret tests were: *Halicystis* ++, *Valonia* +, controls of each species—.

The six *Valonia* cells replaced in sea water gradually died. At the end of 4 weeks, none remained. The *Halicystis* cells, however, were all apparently healthy at the end of 4 weeks.

A repetition of this experiment with 1 per cent proteose in sea water, and an exposure of 2 hours, gave negative Posner biuret tests. The unused cells were observed for 4 days. The *Halicystis* cells remained normal in appearance. This was also true of the *Valonia* cells, with one exception. The largest cell showed pseudoplasmolysis at the end of 48 hours.

3. *The Reactions of Valonia and of Halicystis Cells to Egg Albumen.*  
—No evidence was obtained for the penetration of egg albumen.

Crystallized egg albumen, received through the courtesy of Dr. F. P. Underhill of Yale University, was used in these tests. A solution in sea water was first made. As nearly as could be determined, this was about 1 part egg albumen to 800 parts sea water. This solution was diluted 1:100. The cells were exposed, then, to egg albumen 1 part in 80,000 parts of sea water. This solution gave a strong reaction for albumen in heat tests, Spiegler tests, and Tanret tests. Both the *Valonia* cells and the *Halicystis* cells appeared to be completely tolerant of this solution.

*Valonia* and *Halicystis* cells exposed to a 0.125 per cent solution of egg albumen in sea water for 2 hours, and then washed thoroughly and dried between filter papers, had their vacuole sap tested for albumen by both the Spiegler and the Tanret methods. All tests were negative. One may say, therefore, that if albumen were present, it was in a lower concentration than 1 part per 200,000.

Observations were made of the unused cells for 4 days. Only one *Valonia* cell died.

4. *The Reactions of Valonia and of Halicystis Cells to Diphtheria Toxin.*—There was no evidence for the penetration of diphtheria toxin, nor for the production of antitoxin; but *Valonia* cells were killed, while *Halicystis* cells were highly resistant to its action.

The diphtheria toxin used was prepared at the Massachusetts State Antitoxin and Vaccine Laboratory (No. 46, M. L. D. = 0.006). It had a pH of between 8.0 and 8.2. As before, twelve medium sized *Valonia* cells, or clumps, and twelve medium sized *Halicystis* cells, in separate containers, were exposed indefinitely to the action of this product. The strength of the solution used was 1 cc. of toxin to 150 cc. of sea water, — 1 cc. thus containing 1.0 M. L. D. The *Valonia* cells exhibited a very low resistance. They began to deteriorate within 12 hours. All were dead within 30 hours. In contrast, the *Halicystis* cells were highly resistant. They appeared to be entirely normal during 19 days of observation.

Comparable cells of *Valonia* and of *Halicystis* were exposed to the action of diphtheria toxin in sea water solutions containing 1.0 M.L.D., 0.1 M.L.D., and 0.01 M.L.D. per 1 cc., respectively, for 6 hours. After exposure, the cells were washed carefully in sea water, the vacuole sap carefully withdrawn with tuberculin syringes, using separate syringes and separate 26 gauge gold needles for each species. The samples of

sap were placed in vials, stoppered with cellophane-covered corks, and placed in a refrigerator for transportation to Boston for antitoxin determinations.

The sap and protoplasm from these cells, together with similar extracts from untreated cells as controls, were tested for the production of antitoxin by Dr. W. G. Malcolm of the Massachusetts State Antitoxin and Vaccine Laboratory. 0.1 cc. injections were made intradermally on guinea pigs. Marked erythema, with extreme congestion, appeared immediately. In 24 hours, well circumscribed lesions appeared, showing moderate congestion in the outer zone and necrosis at the center. Extracts from untreated cells produced reactions identical with those from cells exposed to the action of diphtheria toxin. Thus it appears that no antitoxin was formed. One cannot conclude that no toxin passed the protoplasmic membrane, however, for tests of cells injected with toxin were also negative for the production of antitoxin.

5. *The Reactions of Valonia and of Halicystis Cells to Yeast Nucleic Acid.* In this case there was an injurious effect.

The yeast nucleic acid was a concentrated preparation made by Dr. Levene and obtained through the kindness of Dr. Underhill. As in the other experiments, twelve cells, or clumps, of each of the two algae were exposed indefinitely to the action of this material dissolved in sea water. The amount of yeast nucleic acid used was 0.375 gm. per 150 cc. of sea water. To obtain solution, 7.0 cc. of 2 per cent NaOH were required. The solution was then filtered. Pseudoplasmosis began in the largest *Valonia* cells on the 4th day. The small cells remained normal until after the 10th day. By the 18th day, all the cells had disintegrated. The *Halicystis* cells appeared to be normal on the 10th day, but began to deteriorate immediately thereafter. These cells, also, had disintegrated by the 18th day.

No tests were made for permeability.

6. *The Reactions of Valonia and of Halicystis Cells to Edestin.*—In these experiments *Valonia* was more resistant than *Halicystis*.

The edestin preparation, a globulin of the hemp seed, was made by Dr. F. P. Underhill. It was used in 1 per cent solution in sea water. The solution was somewhat milky, and was filtered before using. The filtrate gave a precipitate with acetic acid. Again, twelve cells, or clumps, from each species were exposed indefinitely to the action of the solution. The *Valonia* cells proved to be highly resistant to the action of this material. The largest cell showed signs of disturbance

after 2 days and died at the end of 4 days. At the end of 10 days, two more buds showed signs of disturbance. The remainder were apparently normal, though perhaps showing slight signs of disturbance at the end of 18 days. The *Halicystis* cells, on the other hand, went to pieces at the end of 2 days.

No tests were made for permeability.

As a control for these six experiments, twelve *Halicystis* cells and twelve *Valonia* clumps, similar in size to those used previously, were given identical treatment in cleaning and were placed in sea water for viability observations. The type of container was the same as in the other experiments, and the amounts of water identical. The *Halicystis* cells were apparently normal at the end of 18 days. The *Valonia* clumps, containing forty-three cells in all, remained healthy until the 10th day. Between the 10th and the 18th day, three cells died, but the remaining forty cells were still normal at the end of the observation period.

### *Permeability of Valonia Cells and of Halicystis Cells to Proteose from the Scarlet Runner Bean*

Some evidence for penetration was obtained in this case.

The proteose of the scarlet runner bean used was extracted by Mr. Joseph Cianiurulo of the Massachusetts State Antitoxin and Vaccine Laboratory by the method of E. C. Schneider. The preparation contained 2.475 per cent of nitrogen.

This proteose has the power of agglutinating human erythrocytes. Its strength was tested as follows: A, heated to coagulate protein; B, unheated. Various dilutions were made in isotonic salt solution, and 1.0 cc. of each dilution was added to 1.0 cc. of 1 per cent suspension of human erythrocytes also in isotonic salt solution. The suspensions were allowed to stand in 15 cc. centrifuge tubes in a room temperature of 19°C. The readings given below were made at intervals of 3 hours and of 6 hours, respectively.

Dilution.....	1: 20		1: 200		1: 2000		1: 20,000		1: 40,000		1: 80,000	
Reading.....	A	B	A	B	A	B	A	B	A	B	A	B
3 hrs.	+	-	+	+	++	++	+++	+++	+++	+++	0	+++
6 hrs.	++	-	+++	+++	++++	++++	++++	++++	++++	++++	++++	++++

Apparently normal *Valonia* cells (no *Halicystis* available at the time) were transferred to sea water containing 20 per cent bean proteose (pH 7.0). At the end of 4 hours' exposure, the cells, which still appeared to be normal, were washed carefully, the sap withdrawn into clean, gold-needle tuberculin syringes, and tested against human erythrocytes. Equal amounts of sap and of 1 per cent suspension of fresh blood in isotonic salt solution were used. At the end of 3 hours,

there was definite agglutination of the erythrocytes, with clumps adhering to the sides of the tube. There was no agglutination in the control of salt solution plus blood suspension.

This experiment was repeated, using both *Halicystis* and *Valonia* cells. Controls in this case were untreated sap. After 4 hours of exposure, sap from half of the treated cells was tested against human erythrocytes in dilutions by 10 from 1:1 to 1:10,000. No hemagglutination was observed in any of the tubes.

Of the remaining cells of *Halicystis*, half showed distinct signs of deterioration at the end of 18 hours. The *Valonia* cells were still more susceptible to the treatment given. In 11 hours three-fourths of them were dying.

As it was suspected that some of the *Valonia* cells used in the first experiment did not have normal protoplasmic membranes, similar tests were made on three groups of *Valonia* cells showing increasing amounts of protoplasmic deterioration when examined with dissecting binoculars at a magnification of 200 diameters. These cells showed increasing amounts of penetration by the protease; *i.e.*, they exhibited increased powers of agglutination in direct proportion to the amount of protoplasmic deterioration shown. It was further observed that *Halicystis* cells which showed tiny breaks in the protoplasm under the microscope also gave a plus 4 agglutination test at the end of 6 hours. These tests were made against 1 per cent suspension of fresh blood in isotonic salt solution, using 0.5 cc. of erythrocyte suspension and 0.5 cc. of vacuole sap.

*Valonia* and *Halicystis* cells showing normal protoplasmic membranes under microscopical examination were then exposed for 4 hours to the action of the 20 per cent bean protease solution in sea water, washed carefully, the sap withdrawn as before, and tested. The *Valonia* cells tested + at the end of 1 hour, ++ at the end of 2 hours, with sedimentation at the end of 6 hours. The *Halicystis* cells gave precisely the same tests. Controls against salt solution showed no agglutination at the end of 2 hours, but showed sedimentation at the end of 6 hours. Half of the cells remaining at the end of this last test were replaced in sea water and observed for viability. There were no deaths at the end of 3 days.



## SUMMARY

From the results of these tests it is clear that both *Halicystis* and *Valonia* have a high degree of tolerance for animal peptone, and a very high degree of tolerance for animal proteose and for egg albumen. The products of bacterial growths fostered by these proteins have a deleterious effect upon both species of algae; but, if it were possible to prevent bacterial growth entirely and at the same time supply proper food, it is probable that *Halicystis* and *Valonia* would show normal growth indefinitely in the presence of these three colloids. This is not true where exposure is made to yeast nucleic acid dissolved in sea water containing 0.00093 gm. per cc. of NaOH. *Valonia* is markedly less tolerant of this medium (perhaps of NaOH rather than the colloid used) than *Halicystis*. Such differential effects, however, reach a high point in the case of the solutions of diphtheria toxin and of edestin. *Halicystis* has a very high tolerance for diphtheria toxin, and *Valonia* a very low tolerance. In the case of edestin, the relationship is reversed. Here *Halicystis* has a very low tolerance, and *Valonia* a very high tolerance. In fact, it may be said that diphtheria toxin has no appreciable effect upon *Halicystis*, and edestin a very slight effect upon *Valonia*; while diphtheria toxin is extremely toxic to *Valonia*, and edestin is extremely toxic to *Halicystis*. We can offer no suggestions, at present, as to the way in which these effects are produced.

It is probable that the very thin protoplasmic layer<sup>3</sup> of these species, which is certainly no thicker than  $8\mu$ , is sufficient to obstruct the passage of proteins having large molecules, like egg albumen, with a degree of efficiency that is extraordinary. In the tests we have reported, areas of from 20 sq. cm. to 40 sq. cm. have been submitted to the action of a relatively high concentration of egg albumen for several days without permitting the passage of sufficient amounts to give definable tests either with Spiegler's or with Tanret's method,—presumably less than 1 part in 250,000.

In the tests of the proteins having much smaller molecules (though

<sup>3</sup> Pseudoplasmolized, but turgid, cells of *Valonia* and of *Halicystis*, when subjected to the action of egg albumen in sea water (1:800) for 2 hours, showed the presence of albumen in the cell sap (Spiegler and Tanret). It thus appears that small quantities of albumen pass the cell wall.

the size may not be the explanation), there is some probability that the membranes exhibit a little permeability. The peptone and the proteose of animal origin, or biuret-positive substances<sup>4</sup> derived from them, apparently pass the protoplasmic membranes occasionally in quantities sufficient to give biuret tests. The most probable case of protein passage, however, was that of the proteose of the scarlet runner bean, where specific detection of less than 1 part per 80,000 was possible. In this instance the proteose appeared to pass membranes that were healthy and were functioning normally. But since the cells of the algae had to be destroyed in making the tests, one cannot maintain this point. All one can say is that protein passage was indicated in carefully examined cells of both species, where no breaks in the protoplasmic membrane were discernible, and where samples of the treated cells behaved normally after treatment.

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<sup>4</sup> The presence of non-protein, biuret-positive substances is not excluded; but the method of preparation of the products used in the experiments is such that the presence of detectable amounts is improbable.



# THE REACTIONS OF HALICYSTIS AND OF VALONIA TO INJECTIONS OF CERTAIN PROTEINS

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During the months of February and March, 1932, the writers injected several hundred specimens of *Halicystis Osterhoutii* Blinks and Blinks, and of *Valonia macrophysa* Kütz with solutions of certain proteins, with the dual purpose of learning the tolerance of these algae to the presence of proteins in the vacuole and of determining whether antibodies are formed.<sup>1</sup>

After numerous trials, it was found that the following procedure was essential to success in making the injections. Micro pipettes were drawn out, having an end with a diameter of not over 0.02 cm., passing to a diameter of 0.1 cm. at a distance of about 0.6 cm. from the end, and then rapidly enlarging to 0.2 cm. The pipettes were fitted with rubber bulbs, and were inserted quickly to this point of enlargement. Both the cell wall and the protoplasmic membrane were thus penetrated, leaving the end of the pipette in the midst of the vacuole something over half a centimeter beyond the cell wall. With a little practice, this could be done without allowing the liquid from the vacuole to escape around the sides of the pipette or into the pipette,—the latter point being determinable by the constancy of the height of the liquid. After the insertion of the point of the pipette in this manner, it could be withdrawn slightly and the pressure on the rubber bulb increased sufficiently for a definite amount of liquid to be delivered, the displaced liquid of the vacuole escaping around the walls of the pipette. When this procedure was followed with due care, the pipette could be withdrawn without the admission of air or the further escape of liquid from the vacuole. It is impossible to say, of course, just how much of the injected liquid took a straight course from the end of the pipette and escaped during the manipulation; but from tests using dyes, the amount is believed to be negligible. It is also believed that the amount of liquid lost after the return of the cells of the algae to sea water was very small.

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<sup>1</sup>This work was done at the Bermuda Biological Station for Research. We wish to express our thanks to the Director, Dr. J. F. G. Wheeler, and to Mr. W. Gleeson for their many courtesies, and for their aid in obtaining material.

The cells remained turgid if properly operated upon; and within 5 minutes after the operation, it took distinct pressure with the fingers to force the cell sap out. Within 24 hours, the wound was completely healed, microscopical examination showing a discernible wound callus of the material of the cell wall underlaid by protoplasm.

The pipettes used were calibrated to deliver 0.02 cc., 0.01 cc., and 0.005 cc. at 20°C. This calibration, sufficiently accurate for the purpose, was done by delivering 0.01 cc. or 0.02 cc. of aqueous gentian violet from a standardized pipette on a dry glass slide, and then calibrating the micro pipettes by the amount delivered that would make a drop of the same size.

The solutions used on *Halicystis* were made in sea water, since the liquid in the vacuoles of this species has about the same salt concentration as sea water. The solutions used on *Valonia* were made in "*Valonia* artificial sap," as determined by Osterhout (1931). This artificial sap consisted of NaCl 2.632 gm., KCl 18.65 gm., and CaCl<sub>2</sub> 0.0944 gm. made up to 500 cc. with distilled water. Controls of both *Halicystis* and of *Valonia* were made by injecting with sea water and with artificial sap, respectively.

Somewhat more than 100 cells of each species were injected, not counting those manifestly injured, before the injection technique was perfected to a point where less than 10 per cent of the controls of *Halicystis* died during an observation period of 1 week. The work reported is concerned only with what was done subsequently.<sup>a</sup>

The cells used varied in volume from 0.25 cc. to 4.0 cc.; but 80 per cent of them had volumes between 0.8 cc. and 1.8 cc., the cells of *Halicystis* averaging a little larger than those of *Valonia*. The quantity of liquid injected into the cells having volumes varying from 2.0 cc. to 4.0 cc. was 0.02 cc., thus giving percentages within the vacuoles ranging from 0.5 to 1.0. The remaining cells ordinarily were given injections of 0.02 cc., 0.01 cc., and 0.005 cc., according to size. The solutions injected, therefore, usually formed between 0.7 per cent and 1.1 per cent of the contents of the vacuole. Occasionally, injections of double these amounts were given.

Solutions of 1.0 per cent Difco "bacto-peptone," which had 2.72 per cent ash, 14.92 per cent animal peptone nitrogen, and 0.24 per cent animal proteose nitrogen—a preparation obtained through the kindness of Mr. H. G. Dunham of the Difco Laboratories—were given to both types of cells. About 50 cells of each species were operated upon. No effect was detectable over a period of observation of 10 days, though slightly more *Valonia* died than with the controls.

Similar injections with Difco "bacto-protone" having 2.18 per cent ash, 12.23 per cent animal proteose nitrogen, and 2.67 per cent animal

<sup>a</sup> The one exception to this statement is the experiment with injections of proteose.

peptone nitrogen, induced different reactions. The *Halicystis* cells were affected but little. About 25 per cent died within 10 days, as compared with 15 per cent in the controls made at the same time; but the remainder appeared to be in perfect condition at the end of an observation period of 2 weeks. The injected *Valonia* cells, on the other hand, all died within 2 days; while 80 per cent of the controls lived. This effect is in marked contrast to the behavior of *Valonia* when exposed to the action of Difco "protone" on the outside of the protoplasmic membrane, the cells being very tolerant to the action of the protease under these conditions.

Three sets of *Valonia* were injected with solutions containing 1 part to 800 (Series A), 1 part to 8,000 (Series B), and 1 part to 80,000 (Series C) of crystallized egg albumen. Injections of artificial sap (Series D) were used as controls. The casualties are shown in the table.

Series	No. injected	No. dying on designated day after injection														No. alive
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	
A	57	2	6	21	10	0	0	0	2	1	0	0	0	1	0	14
B	60	2	4	10	11	10	7	5	2	0						9
C	55	2	3	7	9	5	3	0	5	1						20
D	55	2	4	6	8	4	2	1	2	1	0	0	0	1	0	24

Obviously, *Valonia* is adversely affected by the rough treatment experienced during the injections. The death rate is high. But there appear to be significant differences in the trends of the mortality curves and in the percentage of cells remaining alive during the period of observation in the four series. In Series C (1:80,000 egg albumen) and Series D (control) the results are sufficiently similar so that one may consider them to be random samples of a single population. Traumatic shock is probably the primary cause of death. In Series A (1:800 egg albumen) and Series B (1:8,000 egg albumen) there is an additional cause of death. Presumably this cause is the toxicity of egg albumen in the higher concentrations. It is certain that a relatively high percentage of egg albumen was present in the cell sap, for several tests of dead cells showed that a minimum of 0.5 cc. would show albumen by the Spiegler test and that a minimum of 1.0 cc.

would show albumen by the heat test. It should also be noted that, though in each series about the same number of individuals died on the 1st and the 2nd days, during the next 3 days the number of deaths was thirty-one for Series *A*, thirty-one for Series *B*, twenty-one for Series *C*, and eighteen for Series *D*. A death rate of this type, with a lag of a few days followed by a rapid rise, is the characteristic effect of vegetable albumens such as ricin when injected subcutaneously into laboratory animals. The cells of Series *A* and *B* that remained alive until the end of the observation period may have been more tolerant to the foreign protein, but it is somewhat more probable that they actually retained smaller amounts of the injected material.

The nine cells of Series *B* remaining alive on the 10th day after injection were given a precipitin test in order to find out whether antibodies had been formed. The sap plus protoplasm was syringed out with gold-needled tuberculin syringes, centrifuged for 15 minutes, and the supernatant liquid layered carefully on egg albumen in "artificial sap" 1:800. No trace of a precipitin ring was formed.

The fourteen remaining cells of Series *A* and the 20 remaining cells of Series *C* were reinjected with egg albumen 1 part to 800, for the purpose of determining any possible development of increased tolerance—with or without the production of antibodies, or of hypersensitiveness. The cells of Series *A* were thus reinjected 14 days after the first injection, while those of Series *C* were reinjected 9 days after the first injection. The casualties are shown in the table below, all the records except those of the first few days being taken by Mr. W. Gleeson.

Series	No. injected	No. dying on designated day after second injection																								No. alive
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
<i>A</i>	14	2	0	0	1	1	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	7
<i>B</i>	20	2	1	3	1	0	1	2	2	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	4

Unfortunately, the controls surviving from the first injection were discarded by mistake, and therefore were not available for comparison. Despite this deficiency, it is clear that the records show nothing resembling anaphylaxis after the second injection, and no markedly

increased power of resistance. There is a somewhat higher proportion of survivors in Series A, which had previously had the more concentrated dosage of 1:800 albumen, than there is in Series C, which had had only a dosage of 1:80,000 albumen. There is also the further suggestion, in the death rate frequencies involved, that traumatic shock alone accounts for practically all the deaths, since there is not the sharp rise in mortality after a short "incubation" period that characterizes the effect of vegetable albumens on animals and that is found in our own first injections where concentrated solutions were used. It would be necessary to deal with a much larger population before one could attach much significance to these indications, however, both because sampling errors are highly probable and because the survivors of the first injection may have been subject to selection.

The sap and protoplasm from the surviving cells in Series A and C were shipped from Bermuda to Boston in sealed tubes and tested for precipitin reactions by Dr. W. G. Malcolm of the Massachusetts State Antitoxin and Vaccine Laboratory. Dry egg albumen (Merck) 1 part in 100 parts of physiological salt solution was used as antigen. The sap plus protoplasm centrifuged was used in physiological salt solution dilutions by powers of 2 from 1:1 to 1:512. The tubes were incubated 5 hours at 37°C. There were no precipitin reactions shown either here or in controls of untreated cells.

Eighty-five cells of *Halicystis* were injected with crystallized egg albumen 1 part to 800 parts sea water on March 14. After 24 hours, the sap from six cells was used for an albumen test. The Spiegler test was plus 3. 10 days after injection, the sap and protoplasm from eight cells, centrifuged, were used for a precipitin test with egg albumen in sea water 1:1 as antigen. A plus 1 ring was obtained which remained visible for 60 minutes and then faded; but as the upper layer of sap and protoplasm was slightly cloudy, we are not inclined to conclude that there was antibody formation. Of the 71 injected cells remaining, one died after 48 hours. There were no other casualties during a 34 day observation period. The sap and protoplasm of these cells were shipped to Boston and tested for precipitins by Dr. Malcolm, as described above. The test was negative. A small number of control injections were made on *Halicystis*. Out of twenty cells injected with sea water, one died.



It seems reasonable to conclude from these experiments that *Halicystis* shows a high resistance to traumatic shock and a virtually complete tolerance to egg albumen in the concentration of 1:800 when amounts are injected forming between 0.7 per cent and 1.1 per cent (occasionally 2.0 per cent) of the contents of the vacuole. There is no indication, except possibly in one test, that antibodies are formed as a reaction. It appears probable that practically all the albumen is retained within the tonoplast and does not come in contact with the protoplasm in sufficient quantities to cause disturbance; though it is possible that minute quantities may be broken down and utilized as food, or even that minute quantities, as albumen molecules, may egress through the membrane.

In contrast to *Halicystis*, *Valonia* exhibits a low resistance to traumatic shock. It also shows an apparent tolerance to egg albumen in the concentration 1:80,000. In the higher concentrations of albumen, 1:8,000 and 1:800, however, there is evidence of the toxicity of the injected material which manifests itself by a rapid rise in the death rate on the 3rd, 4th, and 5th days after injection. When the survivors of the first injection are again injected with albumen in the concentration 1:800, there is some indication that the toxic effects are less marked.

*Halicystis* having been observed to have a very high tolerance and *Valonia* to have a very low tolerance to diphtheria toxin when exposed to its presence in sea water, a number of injections were made with sea water (for *Halicystis*) and "artificial sap" (for *Valonia*) containing 1.0 M. L. D. per cc. Of *Halicystis*, twenty-four cells were injected (Series E); of *Valonia*, twelve cells were injected (Series F). The casualties were as follows:

Series	No. injected	No. dying on designated day after injection										No. alive
		1	2	3	4	5	6	7	8	9	10	
E	24	0	0	20	2	0	0	0	0	0	0	2
F	12	0	0	12	—	—	—	—	—	—	—	—

The figures in this table show, in a marked degree, the delay in the fatal action of diphtheria toxin which is so characteristic of its effect upon susceptible animals.

It is evident that something occurred here that was far more definite and striking than our usual experience with injected cells. *Valonia* is susceptible to a rather high death rate from traumatic shock, as shown by our other records. But it was the common experience to have about 5 per cent die from acute shock during the first 24 hours, and then to have a rise in the death rate between the 3rd and 5th day, at which times the death rates ranged from 10 to 40 per cent. In this case, however, sudden death overtook the entire population on the 3rd day.

A similar situation is evident in the case of *Halicystis*. *Halicystis* is highly resistant to traumatic shock, as witness the 71 cells injected with egg albumen, of which only one died in 34 days. In contrast, twenty out of twenty-four *Halicystis* cells died suddenly on the 3rd day after injection, and two more were found to be dead on the morning of the 4th day. It is true that two of these injected cells remained alive at the end of the 10th day (when discarded); but one cannot be certain that they were exposed to the same conditions. It is possible that they were tolerant to the diphtheria toxin, but it appears to be more probable that the injected material was lost.

There was not sufficient *Halicystis* material available to make a more extended test of the effect of this preparation. It was possible, however, to make a second test on *Valonia*. Accordingly, 92 cells of *Valonia* were injected with diphtheria toxin dissolved in "artificial sap" in the same proportion as before; *i.e.*, 1 cc. of solution contained 1 M. L. D. of toxin. The results for the first 10 days were as follows:

No. injected	No. dying on designated day after injection										No. alive
	1	2	3	4	5	6	7	8	9	10	
92	1	3	5	3	45	0	7	3	0	3	22

From the 11th to the 16th days, inclusive, there were five casualties,—one each day with the exception of the 12th day. Subsequently there was but one casualty from the 17th to the 40th day.

Here, again, there was an extraordinary rate of death during the first few days, culminating in the death of half the population on the 5th day. The death rate peak's coming on the 5th day instead of the 3rd day can possibly be ascribed to the fact that the air temperature

was several degrees lower than it was when the first series of *Valonia* was injected.

There were sixteen cells remaining alive at the end of 40 days of observation. It is, of course, possible that these cells received no toxin; but we are not inclined to accept this explanation, (a) because they were the last cells injected, and the technique used was the best we were able to devise, and (b) because all the cells, including those which finally survived, gave definite evidence of "sickness" between the 4th and the 8th days. It is more probable that the surviving cells received smaller doses,—that is to say, sublethal doses of the toxin. It is known that the cells dying on the 5th day received quantities of toxin detectable by chemical means, for extracted sap gave definite biuret reactions, something that uninjected cells never do.

The sap and protoplasm from these sixteen cells—and also sap and protoplasm from *Valonia* (two injections) and *Halicystis* (one injection) injected with egg albumen—were brought to Boston and used—after centrifuging—by Dr. Malcolm of the Massachusetts State Antitoxin and Vaccine Laboratory for intradermal injections into guinea pigs. The untreated saps of *Halicystis* and of *Valonia* were used as controls. 0.1 cc. injections were made. There was immediate reaction. A marked erythema appeared which had the appearance of extreme congestion. After 24 hours, a lesion was observable with moderate to severe edema. The lesions were well circumscribed, with moderate congestion of the outer zone, and necrosis at the center. All the tests, including controls, were similar. Thus, while one can say that the sap from the vacuoles of *Halicystis* and from *Valonia* produces marked necrotic lesions when injected under the skin of normal guinea pigs,—reactions that can hardly be attributed to the amount of salts contained,—there is no evidence that diphtheria antitoxin was produced as a reaction to the injections of diphtheria toxin. One cannot determine from these tests, in fact, whether or not the surviving *Valonia* cells injected with diphtheria toxin did, in actual truth, receive it.

#### SUMMARY

It is shown (1) that *Valonia* and *Halicystis* cells exhibit varying degrees of tolerance to injections of animal peptone, animal proteose,

crystallized egg albumen, and diphtheria toxin; (2) that *Valonia* cells display decreased tolerance to egg albumen in increasing dosages, although *Halicystis* is completely tolerant of the highest dosage used; (3) that the mortality curves of *Valonia* injected with egg albumen and of both *Valonia* and *Halicystis* injected with diphtheria toxin show the delayed effect characteristic of laboratory mammals when treated similarly; (4) that *Valonia* cells injected twice with egg albumen exhibit no change in susceptibility to its effects; and (5) that neither species of algae gives evidence of having formed antibodies against the antigens used.



## THE MODIFICATION OF ANTIBODIES BY FORMALDEHYDE

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Interaction of antigen and antibody forms upon the antigen surface<sup>1</sup> a more or less complete film of antibody-globulin held by specific stereochemical forces. The various serological reactions are consequent upon the properties of this surface deposit and the special environing conditions. Thus the antibody-globulin surface deposit under ordinary conditions of test has a surface potential difference below and cohesiveness above the mean critical values for stability, (agglutination occurs (1, 2)); upon the antibody-globulin surface polymorphonuclear and mononuclear phagocytes tend to spread, (phagocytosis occurs (3, 4, 5)); and the antigen-antibody complex is a good adsorbent for the serum components known as complement, (complement-fixation occurs (6, 7)).

Study of the conditions of formation and the physical-chemical attributes of these antibody-globulin films is then clearly of strategic value in gaining further understanding of the serological reactions. The property of the surface film susceptible of most accurate measurement is the electrokinetic P.D. (zeta potential). In undertaking certain studies on this factor we have been aided by possession of strains of bacteria which themselves have only minimal zeta potentials over a wide range of pH, and therefore afford a minimal risk of confusing the zeta potentials of the films with those of the bacterial surfaces (2, 8).

In the present study antibodies before and after combination with antigen have been treated with formaldehyde, and the effects upon

<sup>1</sup> The term "antigen surface" is used without intended implication as to whether the antigen is in molecular dispersion, in micellae, or forms part of a cell surface, and as to whether or not the reaction is stoichiometric.

specific agglutination and upon the isoelectric points of the antibody films have been determined. The data obtained have yielded evidence of the presence of basic groups in the antibody film upon which substitution (or addition) can be effected. Substitution (or addition) upon these groups in the uncombined antibody is possible without greatly interfering with its specific chemical affinity for antigen. It appears, however, that this reaction is not without effect upon the physical properties of the antibody film.

### *Experimental Methods*

*Bacteria Used.*—In earlier experiments a strain of *Bact. coli* and a strain of *Bact. dysenteriae* (Flexner type) were used; in the experiments graphed or tabulated, *Bact. pullorum* and a non-flagellate typhoid bacillus (Strain 0901). All the bacterial strains were smooth. These strains have only minimal zeta potentials in dilute buffers over a wide range of pH (2, 8). The bacteria were grown in infusion broth, washed, and resuspended in 0.85 per cent NaCl solution.

*Chemicals.*—Merck's reagent formalin; this was neutral to litmus except where specified as made neutral to phenolphthalein. Walpole's acetate buffers were used in M/50 concentration over the range of pH 3.6 to 5.6. The pH values were determined colorimetrically, or in the later work with a quinhydrone electrode, taking the value for 0.1 N HCl as pH = 1.08.

*Glassware.*—Glassware was cleaned in concentrated sulfuric acid-potassium dichromate solution, tap water, and distilled water.

*Sensitization.*—The bacteria were sensitized except when otherwise specified with homologous immune rabbit serum. Given volumes of bacterial suspension in 0.85 per cent NaCl solution were mixed with serial dilutions of antiserum. All dilutions of antiserum were made in 0.85 per cent NaCl solution. The tubes were incubated for 2 hours at 37° and refrigerated overnight. Agglutination readings were made. All tubes were then centrifuged until virtually complete sedimentation had taken place. The supernatant fluids were decanted and the sediments were shaken up in about 3 ml. each of 0.85 per cent NaCl solution. All tubes were again centrifuged and the supernatants decanted. The sediments were again shaken up in a minimal amount of 0.85 per cent NaCl solution and each of the suspensions was divided into two portions. To one portion was added 2 ml. of saline and to the second portion 2 ml. of formalin solution. In Table I, Experiment 5, 37 per cent formaldehyde solution was added; in Experiments 1 to 4, and 6 to 9 inclusive, 18.5 per cent formaldehyde solution was added, and in Experiment 10, approximately 9 per cent. The sensitized bacteria, washed and suspended in 0.85 per cent NaCl, are designated in Table I and in the graphs below as Series A; the sensitized, washed bacteria treated with formalin are designated as Series Ax. Both series were allowed to stand 20 to 30 minutes at room temperature. All tubes were centrifuged and the supernatants decanted. The sediments were shaken up in saline and again centrifuged. The supernatants were decanted.

2 drops of saline from a 1 ml. pipette were added to each tube. The tubes were arranged in a row in a rack with the control tubes of bacteria plus saline without serum, in the center. The rack was shaken uniformly until the control tubes showed even suspensions. The sensitized bacteria resuspended in aggregates, the size and resistance to dispersion of which affords a rough estimate of the cohesiveness of the bacteria. Isoelectric point estimations were then made.

In order to determine whether specific combination between antigen and antibody occurred if the immune serum had been previously treated with formalin, the following procedure was followed in addition to the above. The serum dilutions were made up to a given volume, usually 0.5 ml. To each tube the same volume of neutral formaldehyde solution was added, and allowed to stand 20 to 30 minutes at room temperature. In Experiments 1 to 5 the concentration of HCHO was 37 per cent, in Experiments 6 to 9, 18.5 per cent, and in Experiment 10, 9 per cent. To these tubes were added the bacteria suspended in neutral formaldehyde solution of 18.5 per cent concentration (Series B) or in saline (Series D). (In Experiment 5, Table I, the bacteria were suspended in 37 per cent formaldehyde, and in Experiment 10 in 9 per cent formaldehyde. In Experiment 1 the HCHO was neutral to phenolphthalein.) The tubes were incubated for 2 hours in a 37° water bath and refrigerated overnight. Agglutination readings were made, the tubes were centrifuged, the sediments washed in saline solution, and "resuspension" readings were made as described above. Isoelectric points were estimated.

In a few experiments also a set of serial serum dilutions in saline were made and a suspension of bacteria in formalin solution was added. Such series are designated Series C.

The reversibility of the formaldehyde antibody combination was tested in two special experiments. The isoelectric point of the sensitized HCHO-treated bacteria was estimated as explained below. The bacteria were then washed three times and the isoelectric points estimated after each washing. Under these conditions the washings did not cause appreciable reversion of the isoelectric points toward those of the untreated antibody films. This result does not imply, however, that longer contact of the sensitized HCHO-treated bacteria with saline would not have brought out evidence of slow reversibility.

*Cataphoresis and Estimation of Isoelectric Points.*—The Kunitz modification of the Northrop-Kunitz microcataphoresis cell (9) was used with dark-field condensor and Bausch and Lomb 8 mm., 0.50 n.a., 21 × objective. Three readings were taken for each suspension at the two stationary levels; *i.e.*, 0.21 and 0.79 of the inside depth of the cell. Radio B batteries were used; the applied potential of 125 volts gave a gradient through the cell of about 6.5 volts per cm.

The buffer series were made up so that successive members differed by 0.4 of a pH unit. The cell was first washed with the buffer to be used and then a drop of bacterial suspension in about 1 ml. of buffer was washed into the cell with about 2 ml. of buffer. Two successive buffers were found in one of which the particles migrated to the anode and in the other to the cathode; the isoelectric point was then estimated from the relative mean velocities of the two pH values.



TABLE I

No. of experiment	Name of organism	Serum	Interval since serum drawn	Agglutination titre of serum	Agglutination titre of serum treated with HCHO	Tube No.	pH of isoelectric point				
							A	Ax	B	C	D
1	<i>Bact. pullorum</i> (smooth)	Rabbit 18-84 homologous	6 days	1:256	None or slight	1	5.0	4.3	4.5	4.3-4.2	4.4
						2	5.0	4.3	4.45	4.3-4.2	4.4
						3	5.0	4.3	4.45	4.4	4.4
						4	4.9+	4.2			
2	<i>Bact. pullorum</i> (smooth)	Horse typhoid	6½ yrs.	1:256	None or slight	1	4.95	4.1	4.15-2		
						2	4.9	4.1	3.7-3.8		
						3	4.5	3.6?	3.7-3.8		
						4	4.4	3.6?			
3	<i>Bact. pullorum</i> (smooth)	Cow triple typhoid	6½ yrs.	1:256	None or slight	1	4.9-5.0	4.3	4.25	4.2-4.3	4.25
						2	5.0-5.1	4.3	4.3	4.2-4.3	4.25
						3	5.1	4.35	4.35		4.35
						4	5.05	4.35			
4	<i>Bact. pullorum</i> (smooth)	Rabbit 18-87 homologous	2 wks.	1:4096	1:1024 Prozone	1	5.1	4.35	4.6	4.5	4.5
						2	5.0	4.3	4.45	4.45	4.5-4.4
						3	4.9	4.3	4.4	4.4	4.4-4.35
						4	4.7	4.3-4.2	4.3-4.4	4.4-4.3	
						5	4.8	4.4	4.3	4.35	
5	<i>Bact. pullorum</i> (smooth)	Rabbit 18-84 homologous	4 wks.	1:4096	1:256	1	5.2-5.1	4.4-4.5	4.5	4.4-4.5	
						2	5.0-5.1	4.4-4.5	4.4	4.4	
						3	5.0	4.4	4.4	4.4-4.3	
						4	4.75	4.35	4.2	4.4	
						5	4.7	4.3			

6	<i>Bac. pullorum</i> (smooth)	Rabbit 18-84 homologous	9 wks.	1:1024	1:256	1 2 3 4	5.2+ 5.2 5.2-5.1 4.9	4.45 4.45 4.4 4.3	4.5 4.5-4.4 4.45 4.35	
7	<i>Bac. pullorum</i> (smooth)	Rabbit 42 homologous	7 wks.	1:1024	1:256 Prozone	1 2	5.4 5.3	4.5 4.4-4.5	4.2 4.3	4.2-4.3 4.3
8	<i>Bac. typhosum</i> 0 901 (smooth)	Horse 9391B, Rawlings ty- phoid	5 wks.	1:4096 (Prozone)	1:4096 (Longer prozone)	1 2 3 4	4.6 4.6 4.6 4.5	4.0 4.1 4.0 4.0?	4.1 4.1-4.2 4.1	4.1
9	<i>Bac. typhosum</i> 0 901 (smooth)	Rabbit 7	3 days	1:4096	1:1024 Prozone	1 2 3	5.0 4.9 4.9			4.5 4.4
10	<i>Bac. pullorum</i> (smooth)	Rabbit 18-84	7 wks.	1:4096	1:1024 Prozone	1 2 3	5.1-5.0 5.1-5.0 5.0	4.8 4.85 4.75	4.7 4.7 4.7	

## RESULTS

The essential results are shown in Figs. 1 to 4<sup>2</sup> and in Table I. The isoelectric points of these bacteria when maximally sensitized with rabbit antisera fell in the range pH 5.0 to 5.2. These values are about 0.6 pH unit lower than those found for the sensitizing films formed by antibodies against certain other antigens (3, 11). It appears that the sensitizing antibody films formed upon various antigens may have isoelectric points as in this case similar to, or more alkaline (10, 3, 11), or more acid (8) than those of the normal globulins of the species from which the serum is obtained. This point will be developed more fully in a later paper.

Treatment with formaldehyde under the conditions of these experiments has shifted the isoelectric points of the sensitizing films by about 0.6 to 0.8 pH unit toward the acid side. A shift of this approximate magnitude occurred whether the formaldehyde was allowed to react with the antibody film after its formation on the antigen surface, (Figs. 1 to 4, Series Ax), or whether the antisera were mixed with formaldehyde before combination with antigen, (Figs. 1 to 4, Series B, C, and D).

When the antisera were treated with formaldehyde before combination with antigen, agglutination was consistently inhibited in the highest antiserum concentrations, *i.e.* agglutination prozones appeared; see Fig. 1, Series B, C, and D, Fig. 2, Series B and C, and Table I. Cataphoresis and resuspension both showed that these agglutination prozones were due to changes in the physical properties of the sensitizing films rather than to failure of antigen-antibody combination. For the bacteria so sensitized showed appreciable migration velocities in the cataphoresis cell on both sides of their isoelectric points, and the bacteria cohered in the resuspension reaction, indicating that the sensitizing films were present on the bacterial surfaces and merely altered in their properties by the treatment with formaldehyde. Further analysis of the mechanism by which combination with HCHO affects agglutination, and inquiry as to its possible effect on phagocytosis we have not yet attempted.

The end-titre of agglutination was regularly somewhat reduced

<sup>2</sup> The points in Figs. 1 to 4 are joined by lines merely to aid in reading.

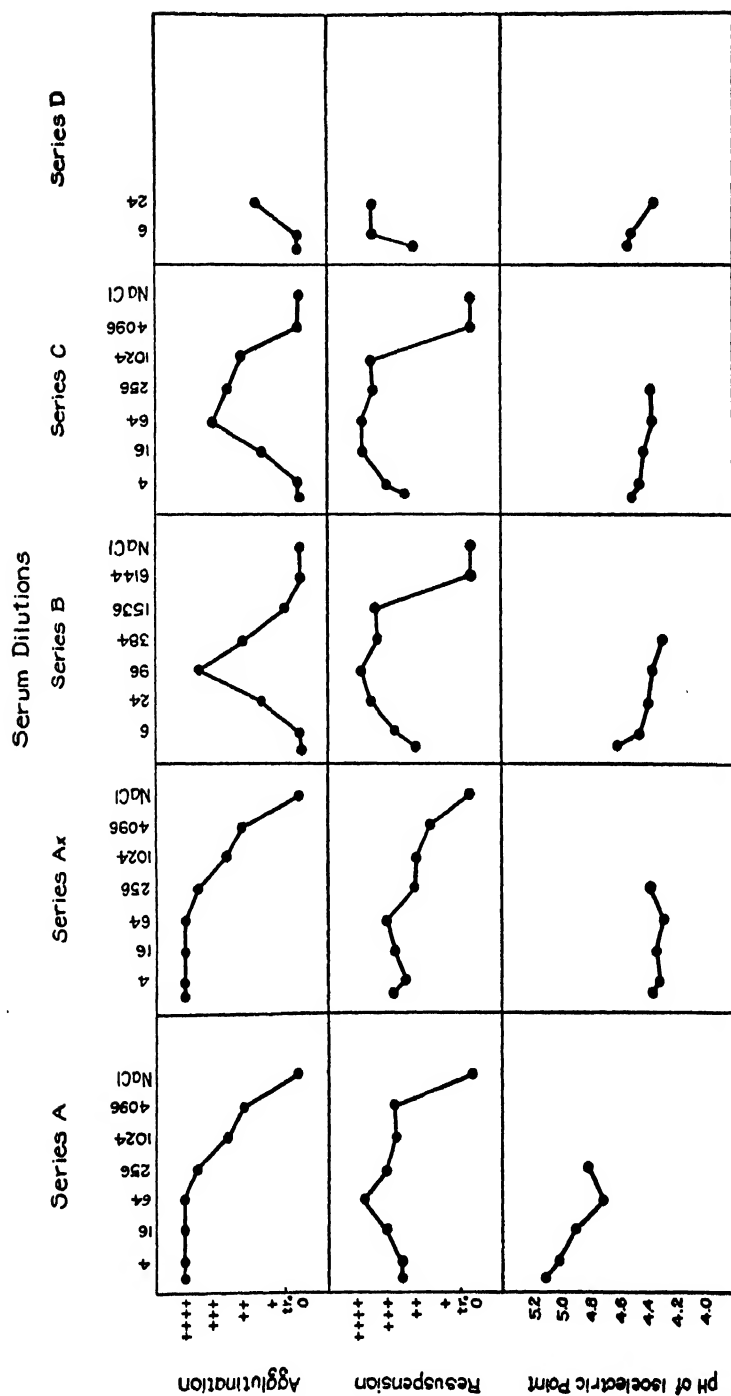


FIG. 1. The effects of formaldehyde treatment on the agglutination, resuspension, and the isoelectric points of sensitized bacteria. Other data relevant to this experiment are given under Experiment 4 in Table I.

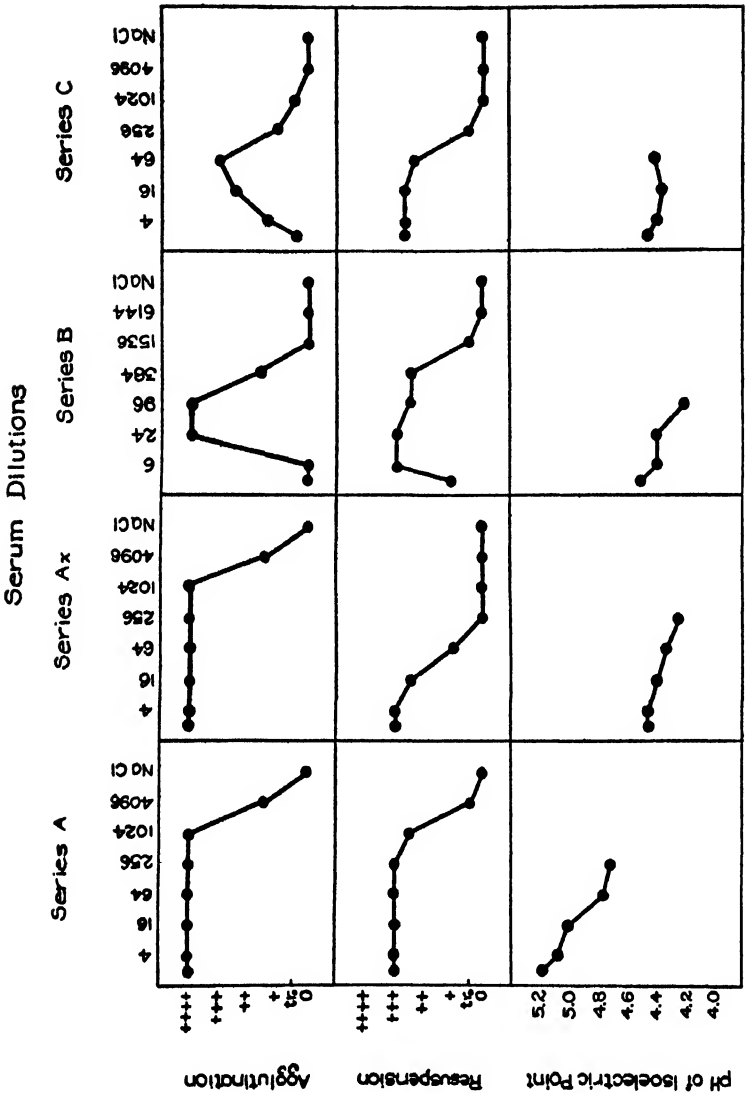


FIG. 2. The effects of formaldehyde treatment on the agglutination, resuspension, and the isoelectric points of sensitized bacteria. Other data relevant to this experiment are given under Experiment 5 in Table I.

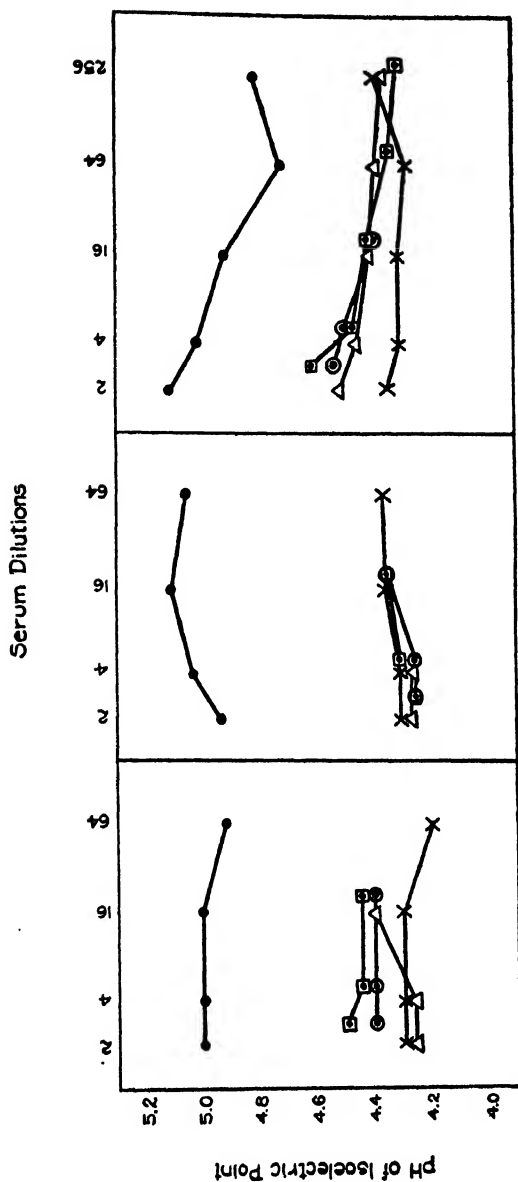


FIG. 3. Effect of formaldehyde treatment on the isoelectric points of sensitized bacteria. The basicity of the sensitizing antibody-protein is diminished in each case by HCHO treatment, as indicated by the shift of the isoelectric point by 0.6 to 0.8 pH unit toward the acid side.

●, Series A, bacteria sensitized with immune serum.

X, Series Ax, bacteria sensitized, then treated with HCHO.

◻, Series B, bacteria sensitized in HCHO, and sensitized with immune serum treated with formaldehyde.

Δ, Series C, bacteria sensitized in HCHO, and sensitized with immune serum.

○, Series D, bacteria sensitized in saline, and sensitized with immune serum treated with HCHO.

Other data relevant to these experiments are given under Experiments 1, 3, and 4, respectively, in Table I.

when the antisera were treated with HCHO before combination with antigen; see Figs. 1 and 2 and Table I. Whether this reduction was due to a slight loss of combining power of antibody for antigen due to HCHO treatment, or merely to the alterations in physical properties is not clear.

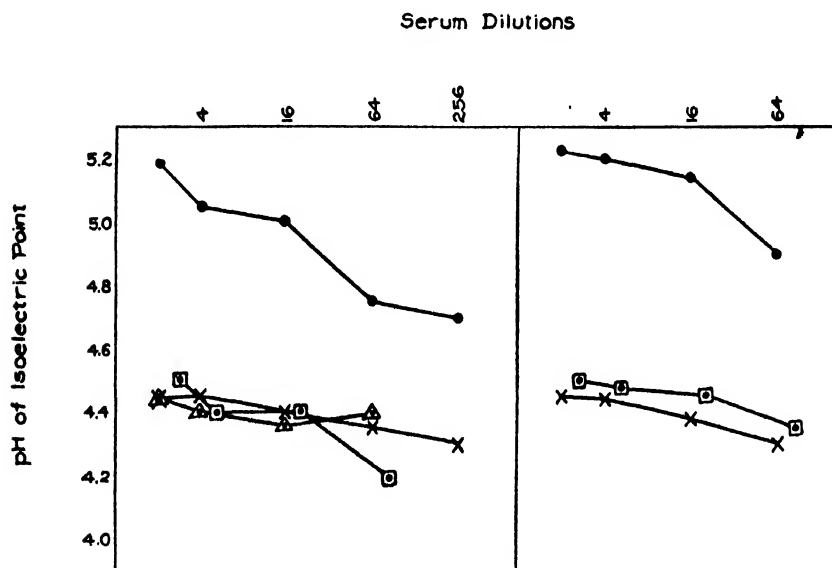


FIG. 4. Effect of formaldehyde treatment on the isoelectric points of sensitized bacteria. Symbols and interpretation as in Fig. 3. Other data relevant to these experiments are given under Experiments 5 and 6, respectively, in Table I.

#### DISCUSSION

Chemical combination of formaldehyde with proteins, involving alterations in the physical properties of the proteins, was demonstrated by Blum (12) in 1896. Blum formalinized a mixture of ovoalbumin and ovomucoid; the resulting proteins were not coagulated by heat and preserved their solubility in water after precipitation by alcohol or acetone. Benedicenti (13) extended these observations in several directions. He allowed gelatin, fibrin, casein, blood serum, and egg albumin to stand in contact with formol and determined titrimetrically a decrease in formaldehyde content of the solution. The "formaldehyde proteins" were in important respects different from the original proteins. Thus gelatin became hardened and insoluble, blood serum underwent gelation, fibrin and casein lost their ability to swell and to be digested. Schwarz (14) performed elementary analyses upon such compounds of proteins with formaldehyde.

Compounds of formaldehyde with amino acids were studied by Schiff (15).

He interpreted the formation of these compounds as a substitution of  $=CH_2$  for  $=H_2$  on the free amino groups. On the basis of Schiff's work, Sørensen (16) elaborated the well known formol titration. More recent investigators have found evidence that the combination of HCHO with amino groups is more complex than the simple substitution of a methylene group for hydrogen.<sup>3</sup> However, there is general agreement in the literature that the principal chemical effect of HCHO on proteins, protein decomposition products, and amino acids is a combination with free amino groups which weakens their basicity.

Kossel (21) and Iodidi (22) have sought to identify the basic groups in proteins which react with HCHO. According to both investigators the  $-NH_2$  group in the  $\omega$ -position in lysine combines with formaldehyde, but the amino group in the guanidine nucleus in arginine is inert. Both agree that HCHO combines with the imino group in the imidazole-ring of histidine; according to Kossel and Gawrilow the imino nitrogen in proline when built into protein is not reactive to formaldehyde.

Pirie and Pinhey (23) and Harris (19) have found evidence of a combination at alkaline reactions between HCHO and the  $-SH$  group of cysteine which results in a perceptible weakening of the acidity of the  $-SH$  group and a shift of the  $pK$  to increased basicity.

The rate of gelation of blood serum in contact with HCHO and the rate of change in solubility in ammonium sulfate of the blood proteins in formaldehyde solutions has been studied by Henley (24). Both changes occur slowly. With a given undiluted serum the rate increases with the HCHO concentration.

The action of 0.5 per cent formaldehyde solution at 37°C. on certain proteins, proteoses, and tryptic digestion products has been investigated by Freeman (25). A decrease in amino nitrogen content as determined by the Van Slyke method was observed after formaldehyde treatment in all the solutions investigated; in no case was loss of amino nitrogen complete, however. Holden and Freeman (26) demonstrated progressive loss of amino nitrogen when amino acids were incubated with 0.5 per cent formaldehyde solutions at 37°C. They showed the combination of meta-protein with HCHO to be to some extent reversible.

Zeiger (27) studied the adsorption of dyes by histological sections of tissue after fixation with formalin as compared with those fixed with alcohol. The reaction at which the tissue elements reversed the sign of their charge as indicated by dye adsorption was shifted toward the acid side by formalin fixation.

Landsteiner and his associates (28) have studied the action of HCHO on the antigenic properties of serum proteins. Rabbit serum was mixed with an equal volume of commercial formalin and allowed to stand at room temperature for 20 hours; the proteins were precipitated with alcohol, washed, triturated and reinjected into rabbits. Antisera were thus produced; these fixed complement specifically in the presence of formalinized rabbit protein but not with formalinized pro-

<sup>3</sup> See Reiner and Marton (17), Bergmann (18), Harris (19), and Levy (20).



tein from the horse, cattle, or fowl. In later work (29) horse sera were formalized and injected into rabbits. The sera of rabbits injected either with native or formalized horse serum precipitated and fixed complement with either native or formalized horse serum but not with the sera of other species.

An extensive investigation of formalized antisera was made by von Eisler and Löwenstein (30). The power of such antisera to neutralize specific exotoxins was unaffected or suffered only slight loss. The amboceptor function (bacteriolytic and hemolytic), and the precipitating function of agglutinins and precipitins were greatly reduced; specific combination of agglutinin and agglutigen occurred, however. Baivys (31) and recently Braun (32) have inhibited the complement fixing, agglutinating, and precipitating action of various antisera by treatment with formaldehyde.

Electrophoresis affords a method of studying proteins adsorbed on surfaces and thus of obtaining data not yielded by ordinary titrimetric procedures. This method has proven peculiarly useful in studying the protein deposited upon bacterial surfaces by interaction with normal and immune serum.

A major difficulty in such studies arises from the fact that the surfaces of many or most bacteria contain materials which are themselves sources of measurable zeta potentials. The isoelectric points of such bacteria sensitized with increasing concentrations of serum fall along ascending curves which rise from the values for the unsensitized bacteria toward those of the sensitizing proteins (2, 11). When strong homologous antisera are used for sensitization such isoelectric point curves may reach levels such that further increase in concentration of serum does not cause further rise in the pH of the isoelectric points (3, 11); whether or not such plateaus represent conditions in which the isoelectric points are due solely to the film-forming substances we do not at present know. At all events, however, normal or heterologous sera rarely sensitize sufficiently to yield such plateau values, and the resulting isoelectric points represent indeterminate values intermediate between those of the substances in the bacterial surface and those of the film-forming substances. Discovery of these bacterial strains with minimal zeta potentials over a wide range of pH therefore affords means of studying adsorbed substances under the many conditions in which the adsorbed film is incomplete.

Electrophoresis has not previously been used, so far as we are aware,

to study the reaction of proteins with formaldehyde. The results here recorded are in essential accord with those obtained by other methods; the protein shows decreased basicity, here evidenced by a shift in the isoelectric point; concomitantly there occur changes in physical properties evidenced in this study by decreased tendency to agglutinate. It is noteworthy that the effects of formaldehyde upon isoelectric points and agglutination were similar whether HCHO treatment preceded or followed combination of antibody with antigen. This result, together with those of von Eisler and Löwenstein, and of Braun, would seem to warrant the conclusion that the HCHO-combining basic groups of antibodies are not involved, or are involved only in a minor degree, in the specific chemical union of antigen and antibody.

#### SUMMARY

Certain strains of bacteria which have only minimal zeta potentials over a wide range of pH, and upon which surface deposits can be formed, afford a favorable means of studying certain chemical and physical properties of the surface deposits.

Films of specific antibody-globulin upon these bacteria possess basic groups which can combine with formaldehyde. Combination of these groups with HCHO under the conditions of the present experiments shifts the isoelectric point of the sensitizing film toward the acid side by about 0.6 to 0.8 pH unit, and reduces the agglutinating tendency of the sensitizing film.

Antibodies may be formalinized before combination with antigen without marked change in their specific combining affinities. The properties of the sensitizing films are similar whether formol treatment occurs before or after the antigen-antibody combination.

The nature of the basic groups has been discussed.

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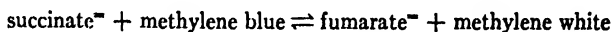
# THE COUPLED REDOX POTENTIAL OF THE LACTATE- ENZYME-PYRUVATE SYSTEM\*

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The reaction:



has been studied by a number of investigators. Quastel and Whetham (1) showed that a definite equilibrium was finally reached when resting *B. coli* were present. Thunberg (2) determined the oxidation-reduction potential of this equilibrium, when the muscle enzyme succinodehydrogenase was the catalyst. Lehmann (3) and later Borsook and Schott (4) worked out the thermodynamics of the equilibrium:



and found that the free energy of the reaction as calculated from the equilibrium electrode potentials agreed very closely with the free energy calculated from the entropies and other physicochemical properties of the reactants. The fact that the calculated and observed values of free energy are so close is proof that the enzyme is a perfect catalyst (Borsook and Schott (4)).

The fact is well established, therefore, that the redox potential of a system such as succinate-enzyme-fumarate, which in itself will establish no definite potential on an electrode, may be correctly determined electrometrically by adding to the system a reversible redox compound having an  $E'o$  in the same range. (The reversible redox compound should have an  $E'o$  such that the logs of the ratios of its

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oxidant/reductant, at final equilibrium, will be less than  $\pm 2$ , as within these limits it acts as a depolarizer and allows the actual potential to be determined.) Presumably, reversible redox compounds can exchange electrons directly with the electrode, whereas the succinate-fumarate system can only oxidize or reduce molecules subject to deformation on the surface of the enzyme (Quastel (5)). The coupled reactions between succinate-enzyme-fumarate, methylene blue-methylene white, and the electrode must, however, occur in a perfectly reversible manner, as from the  $Eh$  of the electrode the correct free energy of the first reaction may be calculated. It would be useful to have a special name for redox potentials determined thus indirectly and the name *coupled redox potential* seems to distinguish it properly from the apparent reduction potential of Conant (6). The *coupled redox potential* may be defined as the potential at which an irreversible system comes into equilibrium with a reversible system when a suitable catalyst is present.

The coupled redox potentials of any two compounds will determine how they will react provided suitable catalysts are present and this will largely determine the transfer of energy, as is implied by Knoop (7) in his article on "The mutual influence of organic compounds in the animal body."

It was with the view of adding a second coupled redox potential to our knowledge that Baumberger (8), in 1929, undertook the study of the lactate-enzyme-pyruvate system. In 1932 Wurmser and DeBoe (10) and Wurmser and Mayer (11) published a determination for this system, finding a coupled redox potential of  $-0.200$  at pH 7.4 and  $37^{\circ}\text{C}.$ , using as catalyst an autolysate of *B. coli*. Baumberger, Jürgensen, and Bardwell (9) presented a paper on this work at the International Physiological Congress in Rome.

### *Method*

The electrode vessels used throughout the work were of a type quite similar to Lehmann's, consisting of a Pyrex U-tube (see Fig. 1) in which the two arms were connected by a capillary filled with agar-agar saturated with KCl. One arm contained the lactate-pyruvate system and the other served as reference cell. The two electrodes, one in each arm, consisted of platinum foil supported by platinum wire, thus eliminating the use of mercury. Oxygen was removed by attaching the stoppers of the two arms to a Y-tube connected to a water vacuum pump having a

nitrogen tank in the circuit. By intermittently evacuating and building up the pressure with nitrogen the oxygen was finally flushed out and the process was ended by evacuation until the vigorous bubbling of the fluid gave place to bumping, at which point the stop-cocks were turned off. The entire evacuation was carried out at 35°C. and required about 5 minutes.

Usually the reactants in the U-tube were added in the following sequence:

1. To the right arm:

0.05 M KH phthalate in 0.15 M NaCl.

Quinhydrone crystals.

2. To the left arm:

4.0 ml. 0.2 M sodium phosphate buffer  $\pm$  NaCl to ionic strength ( $\mu$ ) 0.8.

1-X ml. 0.39 M lactic acid.

X ml. 0.40 M pyruvic acid.

1.0 ml. 0.002 M reversible redox indicator.

2.0 ml. enzyme.

The osmolar concentration of (1) and (2) were equal. The reagents were all kept in the cold room at 3°C.  $\pm$  2°, protected from light, and fresh solutions were frequently prepared. The lactic acid was the usual *d-l* form. Pyruvic acid was freshly distilled at 70° and 16 mm. pressure. Lactic and pyruvic acids were made up to a concentration of 0.39 and 0.40 molar respectively, as determined by titration with standard alkali.

The enzyme preparation was made according to the method of Bernheim (12). Commercial dried yeast was ground to a powder in a coffee mill, treated with acetone, dried, and kept until used in a vacuum desiccator over CaCl<sub>2</sub>. Each week a new enzyme preparation was made by extracting 10 gm. of the dried powder with 100 ml. 6.8 phosphate buffer and 0.1 ml. toluol with occasional grinding in a mortar for a period of 4 hours at room temperature. The extract was then centrifuged at 2500 R.P.M. for 15 minutes, and the supernatant fluid in 20 ml. lots was placed in cellophane (Visking Corporation, Chicago) sausage casings. These were placed in a jar containing 3000 ml. distilled water, which was kept stirred in the cold room. After 24 hours the water in the jar was replaced by fresh distilled water and the dialysis continued for another 24 hours. The contents of the dialyzers, which will be called the "enzyme solution," now showed a negative nitroprusside test for cysteine. The enzyme solution was centrifuged again as above and was placed in a stoppered flask to which a drop of toluol was added. This solution was protected from light and kept in the cold room. Its activity remained high for a period of 2 to 4 weeks but our experiments were carried out with enzyme during the first week after preparation and for each experiment the enzyme solution was recentrifuged immediately before use. Tests of the enzyme solution for cytochrome, lactic acid, pyruvic acid, reducing sugar, and aldehydes were all negative. When no lactate or pyruvate was added, the *in vacuo* experiments showed only slight reduction of methylene blue in many hours.

The vacuum U-shaped electrode vessels, a few minutes after evacuating, were

attached to the frame of a shaker enclosed in an electrically controlled air thermostat at  $32^{\circ}\text{C.} \pm 0.1^{\circ}$ . The shaker imparted a gentle circulatory motion to the fluid in the vessels and caused the electrodes to vibrate. Vessels were so connected that the E.M.F. could be measured while shaking continued.

The E.M.F. of the chain:

Platinum	quinhydrone NaCl KH phthalate	sat. KCl agar-agar,	enzyme lactate pyruvate sodium phosphate indicator	Platinum
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was measured by a Leeds and Northrup potentiometer of the "hydrogen ion" type and an enclosed lamp galvanometer used as null point instrument. Care was taken to avoid polarization of the electrodes. Later a thermionic electrometer was employed to measure the E.M.F. The thermionic electrometer was developed in this laboratory by Mr. R. K. Skow.

The potential of the quinhydrone, NaCl, KH phthalate, was determined before and after the experiment by connecting it to a saturated calomel half cell by means of a saturated KCl agar-agar bridge. This potential was found to remain constant to within  $\pm 0.5$  mv. for a period of 6 hours. If the upper portion of the vacuum electrode vessel is cooler than the lower portion the potential of the quinhydrone half cell continually becomes more negative. This change is due to the *in vacuo* distillation of quinone and its condensation on the cooler surface, where Dr. C. R. Noller collected it and identified it by melting point determination. The fact that quinone may be distilled off from quinhydrone under these circumstances is evidence against the idea that quinhydrone may be a half reduced quinone. It also suggests precautions that should be taken when quinhydrone electrodes are used.

The hydrogen ion concentration of the solutions was determined by means of the glass electrode. Glass electrodes were made of Corning 0.015 glass bulbs sealed to a double walled shank so that deviation film (Kahler and DeEds (13)) effects were obviated and the theoretical difference in potential between different pH's was obtained. The E.M.F. between the glass electrode in 0.05 M KH phthalate in 0.15 M NaCl and a saturated calomel half cell was determined by means of a thermionic electrometer. The glass electrode was then washed and placed in the lactate-pyruvate system from the left side of a vacuum electrode vessel and the E.M.F. against the saturated calomel half cell determined. The difference between the two E.M.F.'s divided by the temperature factor gave the pH difference, which, when added to the pH of KH phthalate, gave the pH of the lactate-pyruvate system. Determination showed no change in pH outside the limits of error, namely,  $\pm 0.02$  pH.

The indicator used was indigo disulfonate (Clark *et al.* (14)) which was more suitable than any other reversible system because its molal electrode potential,  $\bar{E}$ , in the physiological range of pH is approximately + 0.330 or only + 0.014 to

the  $\bar{E}$  of the lactate-pyruvate system according to our determination of it. Methylene blue (Clark *et al.*) had to be used in some of the more acid solutions as the indigo was out of range. Rosinduline (Michaelis (15)) was found to be too negative to be useful.  $\beta$ -anthraquinone sulfonate, (Conant (6)), though, with an  $\bar{E}$  of about + 0.240, falling into range at some ratios of lactate to pyruvate, was discarded because we found that this substance had a marked power of *photo-*

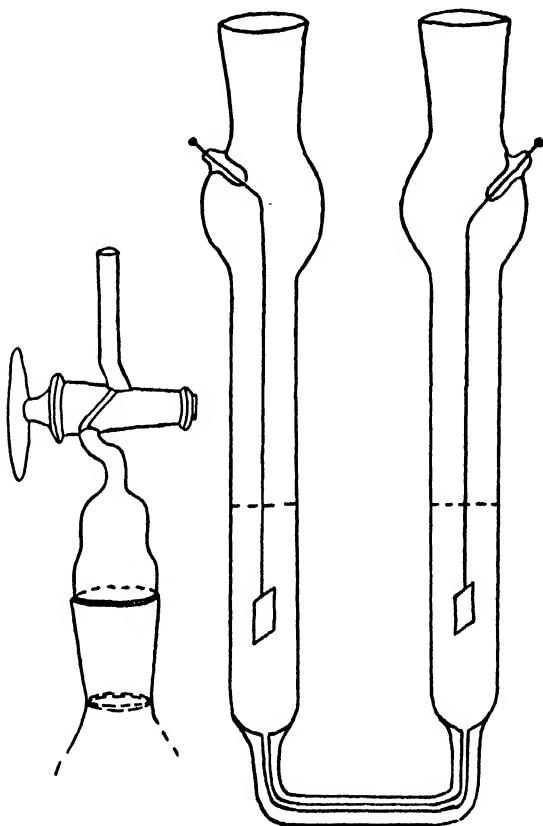


FIG. 1. Vacuum electrode vessel.

*dynamic oxidation* of both lactic and pyruvic acid with the accompanying reduction of methylene blue or indigo disulfonate. This effect is now under investigation by two of us. It is certain that results based on the use of  $\beta$ -anthraquinone sulfonate must be accepted with caution. No measurable difference was noticed when experiments were conducted in light or dark with the other indicators used, but the precaution of minimizing exposure to light was employed so that these experiments were conducted at a light intensity equivalent to twilight.



*Rate of Change of Oxidation-Reduction Potential*

Most of our experiments were made with four vacuum electrode vessels at once. Each vessel had a lactate-pyruvate system at a different pH or different ratio of lactate to pyruvate. The potentials were determined at intervals of about 20 minutes and the results

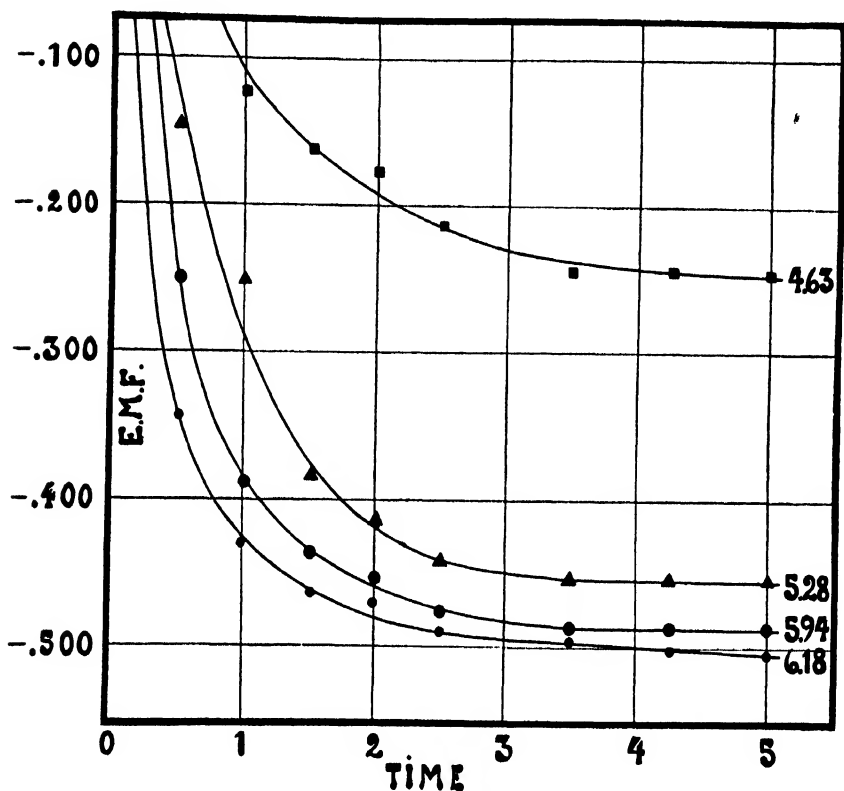


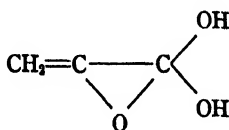
FIG. 2. Potential-time curves at different pH. Time in hours, E.M.F. in volts, pH indicated at right. E.M.F. is the P.D. between the quinhydrone half cell at pH 3.97 and the coupled redox system.

plotted against time. The potentials were found to become very rapidly more negative at first and then the rate of change became gradually slower until finally it became constant or negligible. Fig. 2 shows some typical potential-time curves. It can be seen from the curves that the rate of potential drop approaches zero in the case of relatively acid solutions and reaches a rather low value at pH 6.18.

The final constant rate of change of potential differs according to the pH as shown by the following table:

pH range	Rate of change of potential
	<i>mv./hr.</i>
4.6 – 5.9	<1
6.2	1.5
6.6	2.5
6.7 – 11.8	3.5

This continuous change is probably due to secondary reactions, most likely the formation of condensation products of pyruvic acid, which is accelerated at 37°C. according to Garino and associates (16) and a change in the keto-enol ratio which occurs with change in pH according to Henri and Fromageot (17) and the formation of



It will be necessary to evaluate these three effects before very exact determinations of the equilibrium potential may be made but we have assumed that the effect of the secondary reactions upon the potential may be minimized by considering the rate of change of potential throughout the experiment and selecting as the equilibrium potential the point where the rate of potential change becomes constant. This point is indicated by an arrow on each of the curves in Fig. 3. Values thus obtained are considered *equilibrium potentials* in the remainder of this paper.

#### *Effect of pH on the Equilibrium Potential*

When the ratio of lactate to pyruvate is kept at unity and the pH is varied by means of buffers, the equilibrium potential changes as shown by Fig. 4. This curve of  $E'o$  (Clark *et al.* (14)) changes 0.060 volts per pH unit over the range of pH 5.2 to 7.2, in other words throughout most of the physiological range, and the formula fitting the curve is:

$$E'o - \frac{RT}{F} \ln [H^+] = \bar{E} = 0.316 \text{ at } 32.5^\circ\text{C.} \quad (1)$$

The molal electrode potential (Borsook and Schott (4))  $\bar{E} = 0.316$  is based on twenty-two experiments at sixteen different pH values within the range 5.2 to 7.2. A maximum deviation of  $+0.006$  and  $-0.006$  occurred but the deviations were random.

Outside the range of pH 5.2 to 7.2 we are unable to interpret the change of  $E'o$  with pH. At pH  $< 5.2$  the  $E'o$  changes 0.160 volts per pH unit. To some extent this could be due to the acid dissocia-

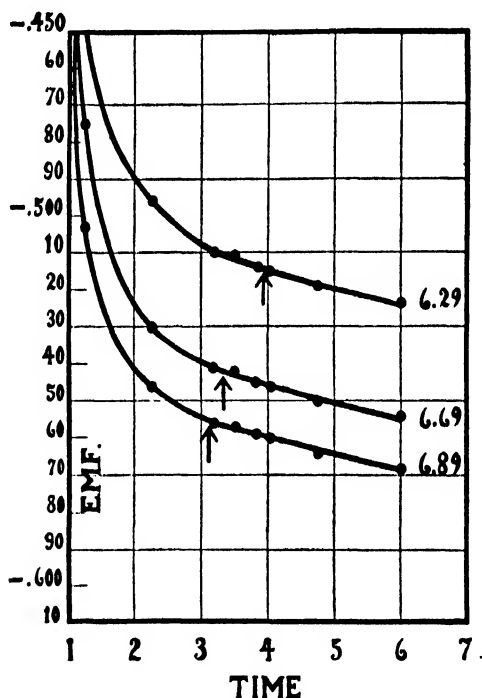


FIG. 3. Potential-time curves. Arrows indicate point where  $\Delta \text{E.M.F.} / \Delta \text{time}$  becomes constant and is considered to be the *equilibrium potential*. (See description of Fig. 2 for further detail.)

tion of the lactic and pyruvic acid, although it is not clear to what degree dissociation of the carboxyl group will affect the oxidation-reduction potential. It is in this acid region, however, that the most marked shift of keto-enol ratio occurs, according to Henri and Fromageot (17), and this shift of ratio could very well affect the  $E'o$ . (Some consideration of the "influence of ionization at points unconcerned in oxidation-reduction" and the "influence of tautomerism" are given by

Clark *et al.* (14).) It does not seem likely that the reductant could have dissociation such as to account for the changes in slope of the  $\Delta E'o/\Delta \text{pH}$  curves from 0.160 below pH 5.2 to 0.060 from pH 5.2 to 7.2 and to 0.030 from 7.2 to 12.0, and we have no other postulates to offer. The data in the pH ranges 2.9 to 5.2 and 7.2 to 12.1 are not as satis-

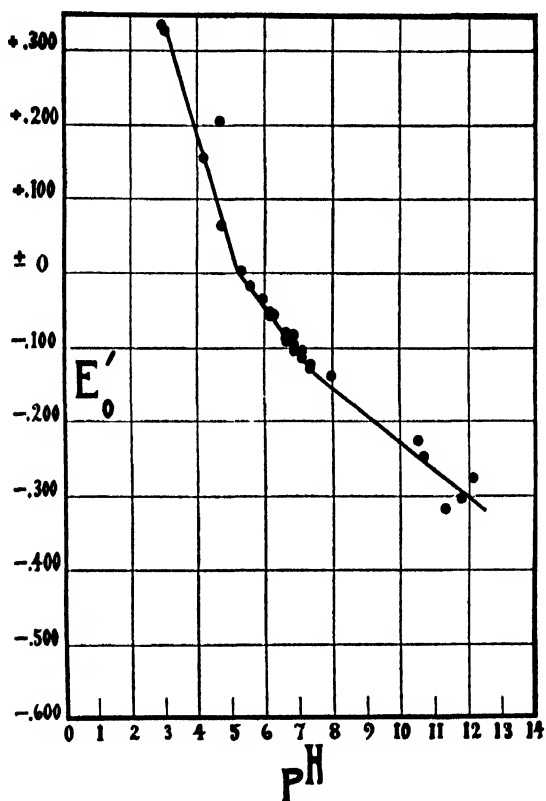


FIG. 4.  $E'o$  - pH curve. Note that  $E'o + 0.06 \text{ pH} = 0.316$  between pH 5.2 and 7.2.

factory as for the region pH 5.2 to 7.2 and we shall confine our attention to this more dependable information.

*The Effect on the Equilibrium Potential of Changes in the Ratio of Pyruvate to Lactate*

The proof of the reversibility of the reaction:



lies in the determination of the effect on equilibrium potential of variations in the ratio of pyruvate to lactate. Experiments were carried out as before with ratios of pyruvate to lactate of 9:1, 5:5, 1:9. Since the stock solution of pyruvic acid had a concentration of 0.4 molar and the lactic acid 0.39 molar, at 32.5°C. the second term of equation (2) would give +0.0291 for the 9:1 ratio and -0.0285 volts for the 1:9 ratio. The expected difference between the two ratios would, therefore, be 0.0576 volts, and observations agreed closely with this, as shown in Table I.

TABLE I

pH	Pyruvate/lactate	0.316 - 0.605 pH + 0.030 log Pyruvate/lactate	Average observed <i>E<sub>h</sub></i>	No. observed
6.80	9:1	-0.066	-0.065	5
6.80	5:5	-0.095	-0.097	9
6.80	1:9	-0.123	-0.122	5

The observed and calculated *E<sub>h</sub>* agreed within 1 or 2 mv. Since the log of the ratio of pyruvate/lactate if multiplied by 0.060, instead of by 0.030 as we have done, would change the values by about 29 mv., there can be no doubt that two electrical equivalents are required for the reaction; i.e., *n* = 2 in equation:

$$E_h = E'_o + \frac{RT}{nF} \ln \frac{[\text{pyruvate}^-]}{[\text{lactate}^-]} \quad (2)$$

Since the observed potentials fit the equation (2) when the ratio of pyruvate/lactate is varied, the process



must be reversible. The process may proceed somewhat as follows: The lactate is oxidized by the indigo disulfonate until the dye is reduced to some degree. When the potential of the partially reduced indigo disulfonate is sufficiently low, the rate of reoxidation of dye by pyruvate will equal the rate of reduction by lactate and equilibrium will have been reached. That lactate is actually oxidized to pyruvate and pyruvate to lactate was not proven by direct analysis but it does not seem probable that equation (2) could hold unless the products

of oxidation of lactic acid and of reduction of pyruvic had the same free energy as pyruvic acid and lactic acid respectively.

### *Recovery of Pyruvate at End of Experiment*

That pyruvate was not further oxidized in the course of our experiments was determined by analysis. Pyruvate was determined by the method of Wieland (18) and no change in this constituent was found in 6 hours. Tests with the Warburg apparatus for the presence of carboxylase (Warburg *et al.* (19)) in the enzyme preparations gave negative results. It may therefore be said that pyruvic acid is not removed during the experiment. An amount of pyruvate equivalent to the dye reduced or oxidized may appear or disappear, but the concentration of dye was kept below 0.01 per cent of the pyruvate, so these changes were within the experimental error of the analytical methods.

### *The Effect of Change in Temperature on the Equilibrium Potential*

The  $\frac{\Delta E}{\Delta T}$  of the process was studied by lowering the temperature from 32°C. to 16°C. after the equilibrium potential had been reached, but the reaction that must go on to reach an equilibrium at a new temperature is so slow that the results have so far been too variable to be of any significance. It is planned to carry out a new set of parallel experiments at two widely different temperatures in order to obtain this data. When such a value has been obtained, it may be used to calculate the heat absorbed during the reaction as by the Gibbs-Helmholtz equation:

$$E + \frac{\Delta H}{NF} = T \frac{dE}{dT}$$

and the increase in entropy ( $\Delta S$ ) of the system could then be calculated:

$$-\Delta H = -\Delta F - T\Delta S$$

### *Thermodynamic Calculations*

The free energy ( $\Delta F$ ) of the process



may be calculated as follows:

$$\begin{aligned}\Delta F &= -NFE \\ &= -2 \times 23057.5 \times -0.316 \\ &= 14,572 \\ -\Delta F &= -14,572\end{aligned}$$

Data are not available for the calculation of the entropy of pyruvic acid, but the ( $\Delta F_{298}^\circ$ ) standard free energy of formation at 25°C. for *d-l* lactic acid (l) is given by Parks and Huffman (20) as  $-124,400$ , and from this value and the  $\Delta F$  of the reaction the  $\Delta F_{298}^\circ$  for pyruvic acid (l) may be calculated as follows:

Lactic ion (1 <i>m</i> )	→ pyruvic ion (1 <i>m</i> )	= $\Delta F_1 = +14,572$
Lactic acid (1 <i>m</i> )	→ lactic ion (1 <i>m</i> )	= $\Delta F_2 = +5,410$
Lactic acid (l)	→ lactic acid (1 <i>m</i> )	= $\Delta F_3 = x$
Pyruvic ion (1 <i>m</i> )	→ pyruvic acid (1 <i>m</i> )	= $\Delta F_4 = -3,709$
Pyruvic acid (1 <i>m</i> )	→ pyruvic acid (l)	= $\Delta F_5 = x^1$
Lactic acid (l)	→ pyruvic acid (l)	$\Delta F = 16,273$

$$\Delta F = \Delta F^\circ \text{ lactic acid (l)} - \Delta F^\circ \text{ pyruvic acid (l)} = 16,273.$$

$$\Delta F^\circ \text{ pyruvic acid (l)} = -124,400 - (+16,273) = -108,127.$$

This is to be considered as merely an approximation, as in the calculation the free energies of dilution  $X$  and  $X^1$  are considered equal, since no adequate data are available for pyruvic acid (Massol (21), Simon (22), Meyerhof (23)).

The free energies of dissociation were calculated as follows:

$$\Delta F_2 = RT \ln Ka_i \text{ where } pKa_i = 3.85 \text{ (Clark (24), p. 678).}$$

$$\Delta F_4 = RT \ln Ka_p \quad " \quad pKa_p = 2.65 \text{ (Barmore (25), p. 41).}$$

## DISCUSSION

That the system lactate/pyruvate is irreversible in the absence of a suitable enzyme was shown by Barmore (25), who, working in the absence of enzymes, found that lactic acid is not oxidized by quinhydrone but is oxidized by  $\text{KMnO}_4$  while pyruvic acid is not reduced by reduced 1-naphol, 2, sulfonate, nor by  $\text{TiCl}_3$ . We have found that lactate + pyruvate + enzyme does not establish a measurable potential on a bare platinum electrode except through the intermediary of a reversible system of suitable  $E'o$ , as for example indigo disulfonate, in the presence of which the coupled redox potential (as defined on page 962) is established.

The fact that this coupled redox potential obeys equation (2) proves that the system is reversible in the presence of the lactic acid dehydrogenase. Wurmser (27) and coworkers have also shown that this is the case by essentially the same method. These investigators added lactate to oxidized dye and pyruvate to reduced dye and followed the

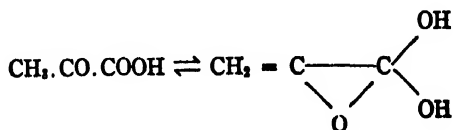
change in potential electrometrically. The enzyme present was Stephenson's (26) preparation of autolysed *B. coli*. The reduced dye (phenosafranine,  $E'o - 0.255$  pH 7.4) was oxidized by pyruvate (but not by lactate) and oxidized dye (cresyl violet  $E'o$  pH 7.4  $-0.175$ ) was reduced by lactate. The potentials of the two vessels approached each other but did not meet because the dyes did not sufficiently poise the electrode in the region of equilibrium.

When, after 24 hours, the reduction of cresyl violet by lactate had ceased, pyruvate was added until the ratio of pyruvate/lactate was 7 (Wurmser (27)). The  $Eh$  at  $37^\circ$ , pH 7.5, was then  $-0.165$ ,  $rH_2 = 9.34$ , from which Wurmser calculates:

$$K = \frac{[CH_3.CO.COO^-]}{[CH_3.CHOH.COO^-]} [H_2] = 7 \times 10^{-9.34} = 10^{-8.40}$$

$$\Delta F = -RT \ln K = 12,040$$

This  $\Delta F$  is 2,500 calories less than the value we obtain and the  $\bar{E}$  0.060 volts less than our determination. A correction for the  $5^\circ C$ . temperature difference might increase this discrepancy by a few millivolts; and it is quite possible that the pH was much greater than Wurmser indicates, because no statement is made regarding pH determinations except that in certain experiments 1 cc. of 7.4 phosphate buffer is added to 1 cc. *B. coli* autolysate, 1 cc. M/20 Na pyruvate, and 1 cc. M/1000 dye. Such a mixture could very well change pH, especially toward greater alkalinity. In all of our experiments the pH was determined at the end of the experiment with all of the reactants present and definite ratios of pyruvate/lactate were present from the start. Fromageot, Pelletier, and Ehrenstein (28) present evidence that the equilibrium:



shifts to the right at pH  $> 5.2$ . A difference in the proportion of this reactive compound, resulting from differences in methods of preparation, might account for our lack of agreement with Wurmser (27); *i.e.*, we may each be determining the  $\Delta F$  of a different compound.



Although some discrepancy exists between Wurmser's data and our own, they both offer proof that the system lactate  $\rightleftharpoons$  pyruvate is reversible.

Haldane (29) has pointed out that the regulatory mechanism that governs the process of cellular oxidation is still unknown. Understanding of this mechanism may be gained through knowledge of the coupled redox potentials of other metabolites, for the  $Eh$  at which the cell is poised may determine the ratio of reactants that can exist. Wurmser (30) has already made some progress in this direction.

Such a calculation can be made for the ratio of fumarate/succinate that can exist in muscle at  $37^\circ$ , assuming the pH 6.8, and assuming that muscle is poised at  $Eh = 0.008$  ( $\bar{E} 37^\circ = 0.418$ ) at which methylene blue would be half reduced, the expected ratio of fumarate to succinate based on  $\bar{E} 37^\circ = 0.423$  (Borsook and Schott (4)) would be:

$$\frac{0.423 - 0.418}{0.0307} = \log \frac{[\text{succinate}^-]}{[\text{fumarate}^-]}$$

Therefore,

$$\text{succinate}^-/\text{fumarate}^- = 1.45.$$

Needham (31) found a ratio of 2 in pigeon breast muscle which is a very good agreement. In a similar manner the ratio of lactate to pyruvate in resting muscle would be:

$$\frac{\left(0.316_{\text{m}} + \frac{\Delta E}{\Delta T} 5\right) - 0.418}{0.0307} = \log \frac{[\text{lactate}^-]}{[\text{pyruvate}^-]}$$

which indicates that given sufficient time for the reaction to come to equilibrium, the system would go entirely to the oxidized form and no lactate would remain. The rate of activation of the lactate by the dehydrogenase would be a limiting factor which would permit of wide departures from the equilibrium state. During activity the  $Eh$  of muscle may drop to a low value, possibly within the range of finite proportion of lactate/pyruvate. The fact that some lactate is always present is due to the continuous glycolysis which keeps adding this reductant in competition with the penetration of oxygen into the cell. The oxygen combines with the hydrogen of cytochrome (or other inter-

mediary) reduced by the lactic acid in its oxidation to pyruvic acid. (Haarmann (32) has found that all tissues reduce pyruvate to lactate *in vacuo*.) The pyruvic acid may then be decarboxylated to acetaldehyde and  $\text{CO}_2$  (Wieland (33)). Meyerhof (23) suggests that resynthesis of glucose in muscle goes mainly through the route lactic-pyruvic-acetaldehyde-glucose. It would not seem likely that pyruvate could be reduced to lactic acid in the cell in view of the coupled redox potential of this system and, therefore, the direction of Meyerhof's synthesis is given support by these thermodynamic considerations.

### SUMMARY

1. The term "coupled redox potential" is defined.
2. The system lactic ion  $\xrightleftharpoons{\text{enzyme}}$  pyruvic ion +  $2\text{H}^+$  +  $2e$  is shown to be reversible (when the enzyme is lactic acid dehydrogenase) and its coupled redox potential between pH 5.2 and 7.2 at  $32^\circ\text{C}$ . is:

$$E'o = 0.316 + \frac{RT}{F} \ln [\text{H}^+] + \frac{RT}{2F} \ln \frac{[\text{pyruvate}^-]}{[\text{lactate}^-]}$$

3. The free energy of the reaction:



4. The standard free energy of formation ( $\Delta F_{298}^\circ$ ) of pyruvic acid (1) is estimated at  $-108,127$ . This is merely an approximation as some necessary data are lacking.

5. The importance of coupled redox potentials as a factor in the regulation of the equilibrium of metabolites is indicated.

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# SWELLING OF ERYTHROCYTES IN SOLUTIONS OF AMMONIUM SALTS

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Besides the hydrogen ion, as is well known, the ammonium ion is the only cation capable of passing through the membrane of erythrocytes. Possibly the mechanism of this is a simple diffusion; possibly, as assumed by Jacobs (1926-27), it consists in a diffusion of  $\text{NH}_3$  into the erythrocyte, followed by an exchange of hydroxyl ions from the corpuscle for the anion with which the ammonium ion was combined in the surrounding fluid.

If the former possibility holds good, the rate of diffusion of ammonium salts into the red corpuscles may be estimated by means of the coefficient of diffusion of a single salt through the membrane:

$$\frac{2 U V}{U + V}$$

If the diffusion of the salt is based exclusively upon an exchange of anions, the question becomes more complicated, as there is a definable coefficient of diffusion only when there is the same fall in the concentration of the two anions.

Taking the simple diffusion of the ammonium ion for a working hypothesis, I have tried to work out an equation from which the rate of the swelling of erythrocytes in solutions of ammonium salts may be expressed by a single numerical quantity, which is to be regarded as a relative diffusion constant for the salt in question. Thus it becomes practicable to compare directly the rates of diffusion of various salts and to study the influence of different factors upon the diffusion rates of individual salts.

## *Equation for Swelling of Erythrocytes in Solutions of Ammonium Salts*

Such an expression can be derived from Fick's law of diffusion:

$$(1) \quad \frac{dm}{dt} = Kq \frac{C_v - C_i}{l}$$

( $dm$  is the amount of salt passing through the membrane in the time  $dt$ .  $C_v$  is the concentration of the salt in the surrounding fluid;  $C_i$  is the concentration of the salt within the erythrocytes.  $q$  is the area of diffusion (surface of the corpuscle);  $l$  is the distance of diffusion (i.e., the thickness of the membrane).)

This law applies to gases and to solutions complying with the laws of gases.

In applying this law to the present condition, it is necessary to express  $dm$ ,  $C_v$ , and  $C_i$  entirely by means of the erythrocyte volume and the initial concentration of the surrounding fluid, as for the sake of simplification it is desirable to use volumetric determinations exclusively.

We start with the assumption that the laws of gases apply to this condition, and that there is no restriction to the applicability of the van't Hoff-Boyle-Mariotte law:  $p_0(v_0 - x) = p_1(v_1 - x)$ . ( $x$  is the dispersion phase of the erythrocytes, which takes no part in swelling or shrinking.  $v_0$  and  $v_1$  are the volumes of the blood corpuscles in solutions with the osmotic pressures  $p_0$  and  $p_1$  respectively.)

We further take it for granted that the surface of the erythrocytes does not change during the swelling and, consequently, that  $q$  and  $l$  can be put down as constant and can be included in  $K$ , a diffusion constant. By this method,  $K$  is only of relative value, and one does not claim it to be more.

Fick's law is then expressed according to the formula,

$$(2) \quad \frac{dm}{dt} = K (C_v - C_i).$$

First we shall study the conditions in a pure solution of an ammonium salt. Here the volume of the erythrocytes is  $v_0$  at the beginning of the experiment (after a possible instantaneous change in the water content if the solution is anisotonic). As there is no ammonium salt within the corpuscles, it will enter them by diffusion. This diffusion of the salt is followed by an absorption of water, so that  $C_v$  remains constant, as the amount of water absorbed is exactly the same as contained  $m$  mol salt when  $m$  is the total amount of diffused salt. We shall let  $v$  represent the volume of the erythrocytes at the time  $t$ . During the time  $dt$  there is a diffusion of  $dm$  mol salt into the erythro-

cytes, together with that amount of water which corresponds to  $dm$  mol in the surrounding fluid;  $dv = \frac{dm}{C_v}$  liter, as the surrounding fluid contains  $C_v$  mol salt per liter of solution. Then we have:

$$(3) \quad C_v \frac{dv}{dt} = K (C_v - C_i).$$

$C_i$  is determined partly by the equation  $C_i = \frac{m}{v-x}$  (in which  $x$  is the dispersion phase of the erythrocytes), and partly by the equation  $v - v_o = \frac{m}{C_v}$ ; that is,  $C_i = C_v \frac{v - v_o}{v - x}$ . Thus the differential equation for the diffusion is:

$$\frac{dv}{dt} = K \left( 1 - \frac{v - v_o}{v - x} \right), \quad K dt = \frac{v - x}{v_o - x} dv.$$

By integration this gives:

$$KT = \frac{1}{2} \frac{(v - x)^2}{v_o - x} + C.$$

$C$  can be eliminated;  $v = v_o$  when  $T = 0$ . This gives:

$$(4) \quad KT = \frac{1}{2} \frac{(v - x)^2 - (v_o - x)^2}{v_o - x}.$$

To test the validity of the expression experimentally it is sufficient merely to place the corpuscles in a solution of an ammonium salt and measure their volume from time to time.

As is evident from the equation—and from experiments—the corpuscles will keep on swelling till they have absorbed all the surrounding fluid. As a rule, however, they undergo hemolysis before this is accomplished.

The hemolysis may be prevented by the addition of a suitable amount of a non-permeating salt or non-electrolyte. In that case another expression is derived from Fick's law:

As before, the osmotic pressure will remain the same on both sides of the membrane only if it stays constant both outside and inside. This requires that the salt diffusing into the erythrocytes —  $dm$  mol — should take along an amount of water —  $dv$  liter — such that the

solution,  $dm$  mol salt in  $dv$  liter solution, has the constant osmotic pressure:  $p = p_i + p^*_i = p_v + p^*_v$ .  $p_i$  and  $p_v$  are due to the permeating salt,  $p^*_i$  and  $p^*_v$  depend on the non-permeating substance.  $p_v = K^*C_v$ ,  $p_i = K^*C_i$ .

$$K^* \frac{dm}{dv} = p, \text{ or } \frac{dm}{dv} = C_v \frac{p}{p_v}.$$

When substituted in (2), it gives:

$$(5) \quad C_v \frac{p}{p_v} \frac{dv}{dt} = K (C_v - C_i).$$

Correspondingly, the amount of salt ( $m$ ) diffusing into the corpuscles, together with the absorption of the amount of water in  $(v - v_o)$  liter, is given as:

$$(5a) \quad \frac{m}{v - v_o} = \frac{p}{p_v} C_v.$$

The  $m$  mol salt is distributed in  $(v - x)$  liter solution of the inner fluid, giving this the concentration  $C_i$ .

$$(6) \quad C_i = \frac{m}{v - x} = C_v \frac{p}{p_v} \frac{v - v_o}{v - x}.$$

Substituting this value for  $C_i$  in (5) gives:

$$(7) \quad \frac{p}{p_v} \frac{dv}{dt} = K \left( 1 - \frac{p}{p_v} \frac{v - v_o}{v - x} \right);$$

$$(8) \quad \frac{dv}{dt} = K \left( \frac{p_v}{p} - \frac{v - v_o}{v - x} \right).$$

$\frac{p_v}{p}$  decreases with an increase in  $t$ , whereas  $\frac{v - v_o}{v - x}$  increases, as  $x < v_o$ .

Therefore, diffusion and swelling will go on, until  $\frac{p_v}{p} = \frac{v - v_o}{v - x}$ ; this means, as is also evident from the connection between  $C_i$  and  $C_v$  in (6), that  $C_i = C_v$ .

If we conceive the volume of the surrounding fluid as very large in proportion to that of the corpuscles,  $p_v$  may be taken as a constant.

If this be the case, then we have:  $\frac{p_y}{p} = \frac{v_s - v_o}{v_s - x}$ . When this expression is substituted in (8), we have:

$$(9) \quad \frac{dv}{dt} = K \left( \frac{v_s - v_o}{v_s - x} - \frac{v - v_o}{v - x} \right) = K \left( \frac{v_s - v}{v_s - x} \right) \left( \frac{v_o - x}{v - x} \right).$$

By integration, this gives:

$$(10) \quad KT = \frac{v_s - x}{v_o - x} \{ -(v_s - x) \ln (v_s - v) + v_s - v \} + C.$$

The value of  $C$  may be calculated, as  $v = v_o$  when  $T = 0$ . We then have:

$$(11) \quad KT = \frac{v_s - x}{v_o - x} \left\{ (v_s - x) \ln \left( \frac{v_s - v_o}{v_s - v} \right) + v_o - v \right\}.$$

#### EXPERIMENTAL

A great number of experiments has been performed. As permeating salt ammonium bromide has been used. As non-permeating substance either Brinkman fluid or Christensen-Warburg fluid has been added to the system.

Brinkman fluid consists of:

Primary phosphate of potassium (Sørensen).....	2.18 gm.
Secondary phosphate of sodium (Sørensen).....	16.3 gm.
Distilled water, free of carbon dioxide, up to 1 liter.	
$\Delta$ 0.458. pH 7.53.	

Christensen-Warburg fluid consists of:

Oxalate of sodium (Sørensen).....	11.3 gm.
Primary phosphate of potassium (Sørensen).....	0.315 gm.
Secondary phosphate of sodium (Sørensen).....	1.365 gm.
Boiled distilled water up to 1 liter.	
$\Delta$ 0.425. pH 7.35.	

The blood in all experiments was fresh human blood with sodium citrate as an anticoagulant. The temperature was kept constant by means of a water thermostat, in which the flasks with the salt solutions and the blood were immersed. At certain times after the beginning of an experiment, specimens were extracted and centrifugated at a rate of 3500 R.P.M. in Hamburger's cono hematocrit tubes. All volumes are expressed as per cent of a standard volume; namely, the volume of the blood corpuscles in their own plasma.

#### *Calculation of the Diffusion Constant*

Application of Equations 4 and 11 to the  $v - t$  curves obtained offers several advantages. For one thing, it makes practicable a direct comparison of the results from experiments with different con-



centrations of permeating or non-permeating substance. Next, it allows the summing up of the results of an experiment with 10–20 volume determinations into a single value:  $K$ , supplemented by  $v_o$  (in Equation 11, by  $v$ , too).

There is a little difference in the calculation of the constant according to whether Equation 4 is used or Equation 11.

Equation 4 reads:

$$KT = \frac{1}{2} \frac{(v - x)^2 - (v_o - x)^2}{v_o - x},$$

and it is to be employed when the surrounding fluid consists only of permeating substance. It includes three unknowns:  $x$ ,  $v_o$ , and  $K$ .  $v$  and  $v_o$  are calculated in per cent of the volume which the same amount of corpuscles would have in plasma.

The value of  $x$ —dispersion phase of the corpuscles—is set at 50 per cent of the volume in plasma. As all the  $v$  values are estimated in the same unit,  $x$  may as a rule be set at 50 (provided that  $x$  does not change).

The value of 50 for  $x$  is derived empirically. It is the value most fitting for human blood corpuscles in the fluids employed in the present work; that is, when all the values obtained have to be adapted to the expressions found for Boyle-Mariotte's law and Fick's law.

In the application of Equation 4 it is theoretically possible to find the value of  $v_o$  by mathematical deduction from several determinations of  $v$ , as  $(v - x)^2 = 2(v_o - x)KT + (v_o - x)^2$ , but the estimations are not accurate enough for this. Therefore,  $v_o$  has to be determined by extrapolation or by calculation according to Boyle-Mariotte's law. The derivation of  $K$  is the same in both equations; it will be mentioned later.

Equation 11 reads:

$$KT = \frac{v_o - x}{v_o - x} \left\{ (v_o - x) \ln \left( \frac{v_o - v_o}{v_o - v} \right) + v_o - v \right\},$$

and it is employed when the surrounding fluid contains non-permeating substance as well as permeating substance.

With Equation 11 the derivation of  $K$  is more difficult. Here we have four unknowns:  $v$ ,  $v_o$ ,  $x$ , and  $K$ . Here too  $x$  is set at 50. As above,  $v_o$  may be derived mathematically in several ways, but estimations of  $v$  as a rule are not accurate enough for this purpose.

So  $v_0$  has to be determined by extrapolation; and this is practicable in particular if the same system is examined at different temperatures, as this gives a greater number of curves for the extrapolation, and  $v_0$  is not affected by changes in the temperature. At low temperatures the swelling of the erythrocytes is slow, at high temperatures it proceeds rapidly. Thus it is practicable in this way to get a set of curves which all converge towards  $v_0$ .

Finally,  $v_0$  can be calculated approximately when the osmotic pressure of the surrounding fluid is known; this value is substituted in Boyle-Mariotte's law, and  $v_0$  is calculated from this,  $\Delta$  of plasma being reckoned as  $0.56^\circ$ .  $v_0$  is more easy to obtain. The curve will approach asymptotically a finite quantity we here call  $v_\infty$ , although it really ought to be designated by  $v_\infty$ , as theoretically it is never reached. However, the corpuscles will soon attain to a size which differs from the theoretical  $v_\infty$  only so little that this deviation is far within the limit of mean error. Measuring the volume several times after this value is reached will therefore give constant values for the volume, and this will practically be equal to  $v_\infty$ . So when the same volume is obtained a few times in succession, it may be counted as  $v_\infty$ . Theoretically it would be practicable also to calculate  $v_0$  after Boyle-Mariotte's law when the osmotic pressure of the non-permeating substance in the surrounding fluid is known. It happens not infrequently, especially in protracted experiments, that the values for  $v_0$  fail to be altogether constant, as hemolysis, agglutination, and similar processes may prevent an accurate reading. When, on the other hand, the end value is reached early,  $v_0$  proves to be a constant value (Experiment 1).

#### EXPERIMENT 1

15 cc. Brinkman fluid + 10 cc. 2.2 per cent  $\text{NH}_4\text{Br}$  + 0.5 cc. blood, 1 hour old

Time	Volume of corpuscles in per cent of their volume in plasma
5 min.	113
11½ "	115
21 "	117
48½ "	116
19 hrs.	117
24 "	117

The value of  $v_o$  is determined for each concentration of permeating salt, and  $v_o$  and  $v_s$  are determined for each combination of permeating and non-permeating salts.

In each experiment a series of determinations of  $v$  are made at different points of time ( $t$ ). It is now attempted to find a value for  $K$  such that the  $v - t$  curve resulting from the substitution of the values obtained for  $v_o$ ,  $v_s$ , and  $x$  in the equation corresponds as well as possible to the  $v - t$  values obtained.

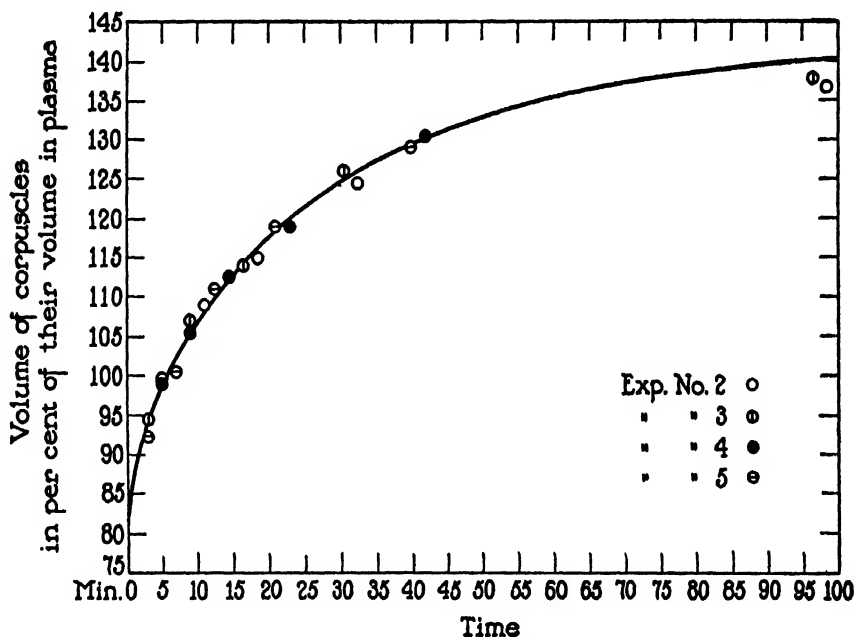


FIG. 1. *Experiments 2-5.*—15 cc. Warburg-Christensen fluid + 10 cc. 4.41 per cent  $\text{NH}_4\text{Br}$  + 0.5 cc. blood. Temperature  $20^\circ$ . Curve drawn: calculated values ( $v_o$  82;  $v_s$  144;  $K$  6.5). Points plotted: observed values.

As the procedure is the same no matter which equation is employed, it suffices for illustration to show its application to the more complicated of the two equations (11).

Four experiments in the same system gave the results presented in Fig. 1.

$v_o$  and  $v_s$  cannot be estimated with sufficient accuracy from these four experiments alone, but summing up the results of all the experiments in this system (a total of 100) gives 144 as the average value for  $v_s$  in

132 determinations, and 82 as the average for  $v_o$ . The latter value is the extrapolation value which fits in the best with the 100 experiments in the system, in which the  $K$  value varies from 1.0 to 30.

A trial is then made with substitution of some values for  $K$  in the equation, with selection of the value that gives a curve covering the greatest number of points—in this example  $K$  was 6.5.

In another series of experiments, one of the systems comprises seven separate experiments carried out at different times. The findings are presented in Fig. 2.

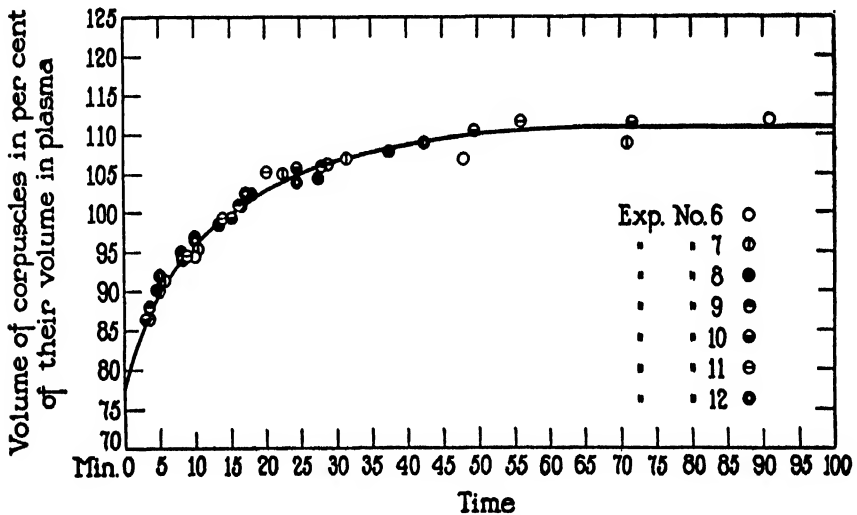


FIG. 2. *Experiments 6-12.*—15 cc. Brinkman fluid + 10 cc. 4.41 per cent  $\text{NH}_4\text{Br}$  + 0.5 cc. blood. Temperature  $20^\circ$ . Curve drawn: calculated values ( $v_o$  77.5;  $v_s$  112;  $K$  6.5). Points plotted: observed values.

Here too  $K$  6.5 was found to give the most consistent curve with  $v_o$  77.5 and  $v_s$  112. All the experiments agree; no other value for  $K$  would apply to any of them.

$K$  may be calculated for every point and an average taken, but the "best possible" curve is almost as exact and much easier to do. Thus, a curve calculated on a formula derived from Fick's law very closely covers a set of points obtained by experiment. I do not claim that this fact has any value in proving the assumptions that we made in deducting the formula. Nevertheless, the size of the surface area of the blood corpuscles may very well be constant within the limits here

adhered to. Jacobs in working out a formula very like mine makes the same assumption.

In numerous experiments with different concentrations of permeating and non-permeating substances, the curve always has covered the points. Of course, the values of  $v_o$  and  $v_i$  have to be altered with the system. All values of  $v_i$  in the different systems follow the van't Hoff-Boyle-Mariotte law closely. The values of  $v_o$  do not always correspond to those expected from this law but there is a certain conformity in the variations in the different systems.

I think then, that these formulae may be used in testing the influences of certain external factors, *i.e.* temperature and hydrogen ion concentration—and the effect of certain substances upon the permeability of the blood corpuscle membrane. For if we carry on our investigations in the same system of permeating and non-permeating salts, any alteration in the size of the diffusion constant  $K$  may be taken as a proof of an alteration in the permeability of the corpuscle membrane.

#### SUMMARY

Two rather simple equations have been derived, which make it possible to express in a single number the result of a series of determinations of the volume of erythrocytes swelling in solutions of ammonium salts.

In all experiments made with several combinations of different concentrations of permeating and non-permeating salts, the curves calculated from the equations have covered the points found by experiment.

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